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71 Applicant: **GENENTECH, INC.**  
460 Point San Bruno Boulevard  
South San Francisco California 94080(US)

72 Inventor: **Heyneker, Herbert Louis**  
501 Roehampton Road  
Hillsborough California 94010(US)

72 Inventor: **Vehar, Gordon Allen**  
14 Maple Way  
San Carlos California 94070(US)

74 Representative: **Armitage, Ian Michael et al,**  
**MEWBURN ELLIS & CO, 2/3 Cursitor Street**  
London EC4A 1BQ(GB)

54 Human tissue plasminogen activator mutants, methods and intermediates therefor, and compositions using such mutants.

57 Biologically active mutant tissue plasminogen activators are disclosed wherein site directed mutagenesis, for example, of a two-chain activation site, especially in the region of residues 270-279, renders said mutants resistant to conversion to the two-chain form.

Particularly preferred mutations are substitution of arginine at residue 275 and/or isoleucine at residue 276.

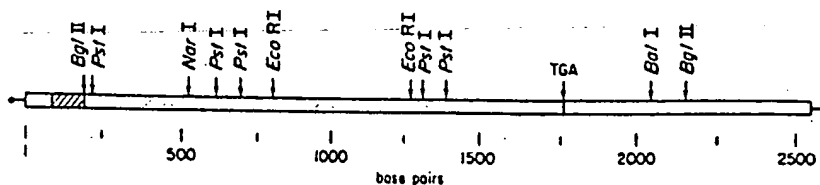


Fig.1.

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HUMAN TISSUE PLASMINOGEN ACTIVATOR MUTANTS ,  
METHODS AND INTERMEDIATES THEREFOR, AND COMPOSITIONS USING  
SUCH MUTANTS

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The present invention is directed to particular novel mutants of human tissue plasminogen activator (t-PA). These mutants, although embraced generically by earlier disclosure, as noted *infra*, are novel, specific derivatives which exhibit activities which defied prediction from the prior research of others on the basic human tissue plasminogen activator molecule or the model serine proteases trypsin and chymotrypsin.

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Human tissue plasminogen activator was first identified as a substantially pure isolate from a natural source, and tested for requisite plasminogen activator activity by Collen, *et al.*, European Patent Application Publication No. 041766, published 16 December 1981, based upon a first filing of 11 June 1980.

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Subsequently, researchers in Assignee's laboratories produced human tissue plasminogen activator, essentially free of proteins with which it is ordinarily associated, via recombinant DNA technology. This work has been recorded in the scientific literature and in European Patent Application Publication No. 093619 published 9 November 1983, based upon a first filing on 5 May 1982. The above patent application (EPO Publication No. 093619) contemplates the production of various human plasminogen activator derivatives, variously modified by

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amino acid substitutions, deletions, additions, or replacements prepared, for example, by site directed mutagenesis of the underlying DNA.

5 As disclosed therein, human tissue plasminogen activator (t-PA) exists in both a single-chain and a two-chain form. The latter is a proteolytic derivative of the former. It has been shown that proteolytic conversion of the single-chain form to the two-chain form occurs during the lysis of a fibrin clot. Rijken, et al. J. Biol.  
10 Chem. 257, 2920 (1982). It is believed that the two-chain form is the agent responsible for plasminogen activator activity, although there have been some initial reports indicating that the single-chain form of human t-PA, Rijken, Ibid, and the single-chain form of procine t-PA, Ranby, et al., Thromb. Res. 27, 176 (1982), may have some activity. See  
15 also Rijken et al., Biochim. Biophys. Acta 580, 140 (1979).

Subsequent investigators, however, have dismissed such reports of single-chain activity as being the result of contamination of these preparations with low amounts of the two-chain form. Andreasen, et al.  
20 EMBO J. 30, 151 (1984); Ichinose, et al. FEBS Letters 175, 412 (1984). Such subsequent reports have reaffirmed the general belief that serine proteases, including t-PA, are expressed as inactive, single-chain zymogens which only become active upon hydrolysis of the protein at a specific site, e.g., arginine at position 275 in the case of t-PA.

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In vivo comparisons of the ability of one-chain verses two-chain plasminogen activator to lyse fibrin clots have been performed using rabbits and dogs. In rabbits, approximately equal potency has been observed for the two forms of this enzyme. Collen et al., J. Clin.  
30 Invest. 71, 368 (1983). When evaluated in a similar model in dogs, however, the one-chain form of plasminogen activator was reported to be slightly less active than the two-chain form. Korninger et al., J. Clin. Invest. 69, 573 (1982). These studies, therefore, indicate that  
35 one-chain plasminogen activator is no better than, and in fact may be less potent than, the two-chain form of plasminogen activator in their

ability to dissolve fibrin clots in vivo.

5       The present application is directed to novel mutants of human  
t-PA, which surprisingly exhibit activity on par with or better than  
the human t-PA first isolated by Collen, et al., (EPO Publication No.  
041766), as well as the t-PA molecules described in the aforementioned  
recombinant patent application. (EPO Publication. No. 093619). In a  
10       particular embodiment, specific mutants covered by the present  
invention include those having certain amino acid substitutions within  
the site surrounding positions 275 and 276 of the human t-PA amino acid  
sequence, occupied respectively by arginine and isoleucine. Certain  
enzymatically active molecules recognize this (these) site(s) (perhaps  
15       together with one or more adjacent amino acids) and functionally  
hydrolyze the bonds after basic amino acids, particularly between  
arginine/isoleucine and lysine/glycine, resulting in two-chain  
material. The two chains remain associated through disulfide bonding  
via cysteine residues. According to this embodiment of the present  
invention, for example, the substitution at these positions with amino  
20       acids other than, e.g., arginine and lysine, serves to produce mutants  
wherein the respective cleavage sites are altered such that two-chain  
human t-PA is not formed in vitro or in vivo, or is formed at a reduced  
rate. Thus, this aspect of the present invention provides mutagenized  
single-chain human t-PA for purposes of testing biological activity.  
25       It has been found that such mutants are rendered immune, or at least  
resistant, to hydrolysis at the 275/276 site and that the resultant  
single-chain human t-PA mutants are unexpectedly on par with the  
activity of the Collen, et al. and/or recombinant t-PA molecules  
described above, in certain biological assays. Furthermore,  
30       indications are that such mutants are less reactive with naturally  
occurring t-PA inhibitors.

In the accompanying drawings:

35       Fig. 1 is a restriction map of the DNA of human t-PA and includes  
5'- and 3'-untranslated regions as well as sequences encoding pre-t-PA.

The speckled area represents the structure gene for t-PA.

Figs. 2a to 2f represent the DNA and amino acid sequences of pre-t-PA including 5'- and 3'-untranslated regions.

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Fig. 3 is the overall scheme used to generate individual clones containing substitutions at position 275.

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Figs. 4 through 8 depict the construction of pXAPPA18 3'ΔX10trpR.

Fig. 9 depicts the plasmid pPADHFR-6 with relevant restriction sites.

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Fig. 10 depicts the plasma protease inhibitor complexes formed by radiolabeled t-PA (left panel) and EIKGG t-PA (right panel) as detected by autoradiography of an SDS-PAGE gel.

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Fig. 11 shows the fibrin binding properties of one-chain t-PA, two-chain t-PA and of the mutated one-chain t-PA (EIKGG).

Fig. 12 depicts a dose-response curve of in vivo clot lysis (EIK is mutated one-chain t-PA; rt-PA is non-mutated t-PA).

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As used herein, "human tissue plasminogen activator", "human t-PA", or "t-PA", denotes human extrinsic (tissue-type) plasminogen activator as produced, e.g., by recombinant cell culture systems, in bioactive forms comprising a protease portion and corresponding to the plasminogen activator otherwise native to human tissue. It will be understood that natural allelic variations exist and occur from individual to individual, demonstrated by (an) amino acid difference(s) in the overall sequence. In addition, glycosylation patterns will depend on the nature of the host cellular environment.

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Human tissue plasminogen activator is a polypeptide which has two functional regions consisting of a protease domain which is capable of

converting plasminogen to plasmin and a kringle-containing domain believed to be responsible for fibrin binding. t-PA therefore includes polypeptides containing these functional domains as part of the overall sequence.

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A "two-chain cleavage site" in t-PA comprises at least the arginine residue at position 275. However, various amino acids adjacent to or within several residues of position 275 are also believed to be a part of the domain recognized by enzymes which convert plasminogen activator to its two-chain form. Thus, replacement of amino acids at positions other than 275 within the domain could result in mutant plasminogen activators which are resistant to conversion to the two-chain form.

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In the particular embodiment, "single-chain plasminogen activator mutant" is a plasminogen activator which is resistant to conversion to the two-chain form. It is characterized by single or multiple amino acid substitutions at the two-chain activation site. As modified, such activation site is not enzymatically recognized, and therefore, not hydrolyzed by enzymes which normally convert plasminogen activator to its two-chain form.

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By analogy to trypsin and chymotrypsin, it is believed that the importance of the formation of the two-chain form of any serine protease is the consequential presence of the free  $\alpha$ -amino group in t-PA at position 276. In this comparison, upon cleavage at arg-275, the  $\alpha$ -amino group 276 would be free to interact with the polypeptide chain in the area of the active site serine of t-PA. The present invention therefore covers any mutation which would interfere with the interaction of such an  $\alpha$ -amino group with the protease active site without diminishing overall activity of the molecule as a whole.

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A variety of methods may be used to induce mutations of underlying DNA so as to prepare the mutants hereof. One such method, illustrated herein as a particularly preferred embodiment, comprises first

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inserting a fragment of the native t-PA gene, containing sequences coding for the region to be mutated, into the replicative form of phage M13mp8 to form M13mp8PA. A synthetic oligonucleotide, complementary to the inserted t-PA sequences but containing one or more nucleotide triplets which code for the amino acid to be substituted, is then annealed to the single stranded form of M13mp8PA to form a double stranded region. This region serves as a primer for DNA polymerase I synthesis of the remaining complementary strand. After replication and identification, the mutant t-PA sequence may be further modified or used to construct a prokaryotic or eukaryotic vector for expressing the mutated t-PA polypeptide.

The above described general method may also be used to mutate t-PA at positions other than the 275/276 and/or 277/278 two-chain cleavage sites, to produce mutated t-PA derivatives falling within the present invention. Such other positions are polypeptide sequences which are susceptible to enzymatic hydrolysis such as trypsin-like cleavage sites which typically comprise arginine or lysine followed by isoleucine, serine, or alanine. Substitution of one or more amino acids within such trypsin-like cleavage site results in mutant t-PAs which resist hydrolysis by trypsin-like proteases. Such resistance to enzymatic degradation during expression and purification as well as during in vivo administration as a pharmaceutical agent results in a t-PA which does not lose biological activity as compared to the non-mutated t-PA. Examples of such trypsin-like cleavage sites within the human t-PA molecule include arginine-alanine (positions 40-41), arginine-serine (positions 27-28), and arginine-serine (positions 462-463).

A. General

Mutated t-PA derivatives hereof are prepared 1) having methionine as its first amino acid (present by virtue of the ATG start signal codon insertion in front of the structural gene) or 2) where the methionine is intra- or extracellularly cleaved, having its normally first amino acid, or 3) together with either its signal polypeptide or a conjugated protein other than its conventional signal polypeptide.

the signal polypeptide or a conjugate being specifically cleavable in an intra- or extracellular environment, or 4) by direct expression in mature form without the necessity of cleaving away any extraneous, superfluous polypeptide. In any event, the thus produced human mutated t-PA, in its various forms, is recovered and purified to a level suitable for the treatment of various vascular conditions or diseases such as myocardial infarct, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion and other thrombotic conditions.

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Human mutated t-PA also has a functional definition in being capable of binding to fibrin and of mediating in vivo or in vitro conversion of plasminogen to plasmin which in turn solubilizes fibrin clots.

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"Expression Vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operably linked to other sequences capable of effecting their expression and which are replicable in the host organisms, either as episomes or as an integral part of the chromosomal DNA.

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"Recombinant host cells" refers to cells which have been transformed with expression vectors constructed using recombinant DNA techniques.

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#### B. Host Cell Cultures and Vectors

The vectors and method disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used such as E. coli B, and E. coli X1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

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In addition to prokaryotes, eukaryotic organisms, such as yeast cultures, may be used. Cultures of cells derived from multicellular

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organisms are the hosts of choice currently. In principle, any such cell culture is workable; however, interest has been greatest in cells from vertebrates, and propagation of these cells in culture (tissue culture) has become a repeatable procedure - see Tissue Culture.  
5 Academic Press, Kruse and Patterson, editors (1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese Hamster Ovary (CHO) cell lines, W138, BHK, COS-7 and MDCK cell lines.

10 Examples which are set forth hereinbelow describe the use of E. coli using the trp promoter system and the use of CHO cells using expression vectors which include the SV40 origin of replication as a promoter. However, it would be well within the skill in the art to use alternative prokaryotic or eukaryotic host cell cultures.

15 C. Methods Employed

1. Transfection

If cells without formidable cell wall barriers are used as host cells, transfection may be carried out by the calcium phosphate precipitation method as described by Graham et al., Virology 52, 546  
20 (1978). However, nuclear injection or protoplast fusion may also be used.

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is  
25 via calcium chloride as described by Cohen, et al. Proc. Natl. Acad. Sci. (USA) 69, 2110 (1972).

2. Vector Construction

30 Construction of suitable vectors containing the desired coding and control sequence employ standard ligation techniques known per se. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

D. Examples

35 1. Construction of M13mp8PABgIII For t-PA Mutagenesis

Human t-PA DNA was obtained from plasmids pPADHFR-6 (also designated pETPFR) and pA25E10. The preparation of these two t-PA plasmids is described in EPO Application Publication No. 093619, referred to above and incorporated herein by reference.

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Plasmid pA25E10 contains sequences coding for the last 508 amino acids of the t-PA gene and 772 base pairs of the 3' untranslated region. This plasmid was digested with SacI and BglII to produce a 744 base pair fragment which was isolated by standard methods as previously described. As can be seen from the known sequence and restriction map of t-PA in Figure 1, this fragment contains the codons for t-PA amino acids 411 through 527 and includes part of the 3' untranslated region.

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Plasmid pPADHFR-6 contains the entire structural gene for t-PA and part of the 3' untranslated region. This plasmid was digested with SacI and BglII to produce a 1,230 base pair fragment which was isolated. This fragment contains codons for the first 410 amino acids of the mature form of t-PA.

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These fragments were ligated together using standard methods and digested with BglII. A 1,974 base pair fragment containing codons for the entire mature t-PA sequence plus part of the 3' untranslated region was isolated. Double stranded M13mp8, (Messing, et al. Third Cleveland Symposium on Macromolecules Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981), p. 143) was digested with BamHI and annealed to the BglII digested t-PA to form M13mp8PABglII. E. coli JM 101 cells (ATCC No. 33876) were transformed with the double stranded replicative form of M13mp8PABglII. The single stranded and double stranded (RF) forms of M13mp8PABglII may be isolated from E. coli JM 101 cells infected with this phage. The single stranded form was used for the site specific mutagenesis of t-PA.

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## 2. Synthesis of Primers for Site Specific Mutagenesis

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The human t-PA structural gene was modified by site

specific mutagenesis to express t-PAs with amino acid substitutions at various positions. Synthetic oligonucleotides were prepared such as by the solid phase phosphotriester method of Crea *et al.*, Proc. Natl. Acad. Sci. (USA) 75, 5765 (1978). The following synthetic primers were prepared and used for such site specific mutagenesis:

	Native Amino	275	279
	Acid Sequence	Pro Gln Phe Arg Ile Lys Gly Gly	
10	Native DNA Sequence	G CCT CAG TTT CGC ATC AAA GGA G	
	Primer 1B8	Gly	
	DNA Sequence	G CCT CAG TTT <u>GGT</u> ATC AAA GGA G	
	Primer 2C9	Glu	
	DNA Sequence	G CCT CAG TTT <u>GAA</u> ATC AAA GGA G	
15	Primer 4A10	Gly Ile	
	DNA Sequence	G TTT <u>GGT</u> ATC <u>ATC</u> GGA GGG CTC	
	TT		
	Primer 3A7	Gly Ile	
	DNA Sequence	G CCT CAG TTT <u>GGT</u> ATC <u>ATC</u> GGA G	
20	Primer 4B3	Glu Ile	
	DNA Sequence	G CCT CAG TTT <u>GAA</u> ATC <u>ATC</u> GGA G	

The amino acid and gene sequence of native t-PA is depicted in the first two lines. The primers have triplets which differ from the native gene sequence at the residue shown. The corresponding amino acid substitution is shown above the triplet coding for that amino acid.

### 3. Site Specific Mutagenesis

The procedure described hereinafter, was used to generate different t-PA clones containing the mutated sequence of the synthetic primers. The general method used is that of Adelman, *et al.* DNA 2, 183 (1983), incorporated herein by reference. The overall scheme to generate each of these clones is presented in Figure 3. M13RF1B8, M13RF2C9 and M13RF4A10 were generated by the use of primers containing mutations for the single amino acids shown. Single standard M13RF4A10,

containing a mutation at position 277, was annealed with primer 3A7 or 4B3 to generate M13RF3A7 and M13RF4B3 respectively. Purified M13 RF DNA from each of these mutated t-PA genes was prepared from *E. coli* JM 101 cells. Subsequently, DNA fragments containing the mutated t-PA DNA sequence were used to construct expression vectors for the mutated t-PA.

50 ng of a synthetic oligonucleotide was phosphorylated for 30 min at 37°C in 10 µl of 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM ATP containing 8 U of T4 polynucleotide kinase. For use as a probe, 400 ng of the synthetic oligonucleotide was phosphorylated as above except that ATP was replaced with 60 mCi [ $\gamma$ -<sup>32</sup>P]-ATP (3000 µCi/mmol) resulting in approximately 50 to 60 x 10<sup>6</sup> cpm/400 ng of 24mer. For heteroduplex formation, 10 ng single stranded M13mp8PABgIII was heated to 95°C (10 min), and slowly cooled to room temperature (30 min) in 40 µl 10mM Tris-HCl pH 7.5, 10mM MgCl<sub>2</sub>, 1mM dithiothreitol containing 10 ng of the phosphorylated primer and 50 ng of *Eco*RI-digested M13mp8PABgIIIRF large fragment. Primer extension was started by the addition of 10 µl ligase buffer containing 2mM ATP, 0.25 mM each of dGTP, dTTP, dCTP and dATP, 5 U of *E. coli* DNA polymerase I large fragment and 400 U of T4 DNA ligase. After 1 hr at 12°C the reaction mixture was used to transform *E. coli* JM101 cells.

Transformation was accomplished by mixing 10 µl of the ligation mixture with 200 µl of competent JM101 cells, followed by incubation for 30 min on ice and 5 min at 37°C. Then 3.5 ml 2YT top agar at 55°C was mixed with 300 µl saturated JM101 cells, 10 µl IPTG (200 mM) and 50 µl Xgal and after addition of the transformed cells plated on 9 cm Petri dishes containing LB with no drugs.

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Colorless plaques were picked and transferred to a microtiter dish containing 100 µl 2YT medium. The inoculated microtiter fluids were stamped on 15 cm diameter LB agar plates overlaid with a lawn of 600 µl JM101 cells in 8 ml 2YT top agar and incubated overnight at 37°C. The formed plaques were transferred to a nitrocellulose disc by

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physical contact for 1 min. The nitrocellulose disc was treated with 0.5 M NaOH, 1.5 M NaCl for 3 min and washed twice with 3 M NaCl-0.5 M Tris HCl pH 7.5 for 15 min and then with 2X SSC for 15 min. Prehybridization mix contains 10 mM Tris pH 7.5, 5 mM EDTA, 0.9 M NaCl, 1X Denhardt 0.5 percent NP40, 100  $\mu$ M ATP, 1 mM sodium pyrophosphate, 1 mM sodium phosphate and 50  $\mu$ g/ml *E. coli* tRNA. 1X Denhardt's contains per liter 200 mg Ficoll, 200 mg polyvinylpyrrolidone, 200 mg bovine serum albumin (BSA; fraction V). The disc was baked at 80°C in vacuo for 90 min. The disc was then incubated for 3 hrs with 6 ml prehybridization fluid in a Petri dish followed by addition of  $5 \times 10^6$  cpm labeled primer and hybridized overnight. Selective washing of the disc was performed with 0.4X SSC at 49°C and after air-drying the disc was exposed to X-ray film. Positively hybridizing clones were further analyzed by dideoxy sequencing. See Aldeman, *Ibid.*

4. Construction of Vectors for Expression of Mutant t-PA in *E. coli* pXAPPA18 3' $\Delta$ x10trpR

The plasmid pXAPPA18 3' $\Delta$ x10trpR plasmid was constructed for use as an expression vector for the various mutated t-PA DNA sequences. The overall scheme used for construction of this plasmid is depicted in Figures 4 through 8. The resulting plasmid is depicted in Figure 8. It contains the trpR repressor gene and a deletion of pBR322 DNA sequences which inhibit plasmid amplification. This deletion, known as XAP deletion, consists of the removal of 641 base pairs of pBR322 DNA sequences between the AvaI and PvuII restriction sites of pBR322 as disclosed by Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology, Vol. 43, 77 (1979) Cold Spring Harbor Press, incorporated herein by reference. The trpR repressor gene compensates for the premature derepression of t-PA expression caused by increased plasmid copy number. Intermediate to the construction of pXAPPA18 3' $\Delta$ x10trpR is the plasmid pPA18 which was constructed as depicted in Figure 4. This plasmid contains the entire pre-t-PA structural gene as well as 5' and 3' on untranslated regions. A trp promoter associated with the t-PA gene and sequences conferring ampicillin and tetracycline resistance are also characteristic of this plasmid.

In order to construct pPA18, four plasmids were used, namely pFIFtrp69, pHKY10, ptPATrpl2 and pPA25E10. Plasmid pFIFtrp69 is disclosed in Goeddel *et al.*, Nucleic Acids Res. 8,4057 (1980). Plasmid  
5 pHKY10 is disclosed in U.S. Patent Application Serial No. 685,521 filed December 24, 1984 which is a continuation of U.S. Serial Number 307,473 filed October 1, 1981 which is a continuation of Serial Number 133,296 filed March 24, 1980 (European Patent Application Publication. No. 0036776). Plasmids ptPATrpl2 and pPA25E10 are disclosed in Pennica, *et*  
10 *al.*, Nature 301, 214 (1983), and in EPO Publication No. 093,619 *supra*.

Generally, the plasmid pFIFtrp69 is digested with PstI and XbaI to produce the 950 base pair fragment designated fragment 1 in Figure 4. The plasmid ptPATrpl2 was digested with XbaI and NarI. From  
15 this the 340 base pair sequence designated fragment 4 in Figure 4 was isolated. The plasmid pPA25E10 was digested with NarI and BglII. From this was isolated the 1604 base pair fragment designated fragment 3 in Figure 4. The plasmid pHKY10 was digested with PstI and BglII to produce a 2900 base pair fragment designated fragment 2 in Figure 4.  
20 These four fragments were ligated and this DNA used to transform *E. coli* cells to give pPA18.

The plasmid pPA18 was isolated and digested with Sau3A followed by treatment with the Klenow fragment of DNA polymerase I to  
25 fill-in the restriction site. The non-circular plasmid was treated with SacI and a 389-base-pair-sequence designated fragment 5 in Figure 5 was isolated. Plasmid pPA18 was also digested with SacI and BamHI. From this the vector fragment 6 was isolated. The plasmid pBR322, Boyer *et al.*, Gene 2, 95 (1977), was digested with EcoRI followed by  
30 treatment with the Klenow fragment of DNA polymerase I. This open-ended DNA sequence was treated with BamHI to produce the 375 base pair sequence depicted as fragment 7 in Figure 5. Fragments 5, 6 and 7 were ligated and this preparation used to transform *E. coli* from which the plasmid pPA183'A was obtained. This plasmid is equivalent to pPA18  
35 except that part of the 3' untranslated region of the t-PA gene has

been removed.

The plasmid pPA183' $\Delta$  was digested with PstI and NarI to produce a 313 base pair fragment designated fragment 8 in Figure 6. This fragment encodes amino acids 8 through 109. Synthetic oligonucleotide fragment 9 has the following sequence:

5' CTAGAATTATGTCTTATCAAGTTATTGCA  
TTAATACAGAATAGTTCAATAA 5'

This synthetic DNA was ligated to the PstI site of fragment 8 to regenerate the arginine codon at position 7 and the first six amino acid condons of the mature t-PA molecule. In addition, a ribosome binding site was positioned 5' to the synthetic N-terminal methionine condon positioned immediately 5' to residue 1 of the mature t-PA amino acid coding sequence. The 5' end of this oligonucleotide contains an XbaI restriction site. Thus, fragment 8 was ligated in the presence of the synthetic oligonucleotide fragment 9 and the mixture treated with XbaI and NarI to give fragment 10 (See Figure 6).

Plasmid pPA183' $\Delta$  was digested with NarI and SacI to produce the 900 base pair sequence designated fragment 11 in Figure 7. This plasmid was also digested with SacI and XbaI to produce vector fragment 12 in Figure 7. Fragment 10, 11, and 12 were ligated and used to transform E. coli from which was isolated pPA183' $\Delta$ X10. A DNA sequence containing the XAP deletion and trpR repressor gene is derived from pFMBtrpR which is disclosed in U.S. Patent Application Serial Number 538,730 filed October 3, 1983 (EPO Publication No. 136907). Briefly, this plasmid was constructed from three plasmids known to those skilled in the art: phGH107, described in EPO Publication No. 022242, published January 14, 1981, was used as a source for the lac inducible promoter; ptrpR3, described in Roeder, et al. Molecular Genetics 176, 361 (1979) was used as the source of the coding sequence for trp repressor; and pFMB1, described in EPO Publication No. 0068693 published January 5, 1983, was used as the source of the coding sequence for the FMD antigen derived from strain A24.

To obtain the trp repressor sequence, ptrpR3 was treated with HaeIII, and the 334 base pair fragment was isolated from a 6 percent acrylamide gel and the isolated fragment was ligated with 16-mer EcoRI linkers having the sequence:

5'CCATAGAATTCTATGG.

To obtain vector backbone and the lac promoter, phGH107 was first digested with EcoRI, and treated with bacterial alkaline phosphatase. The large vector fragment containing the lacUV5 promoter was then ligated to the tailored trpR plasmid using T4 ligase, and the ligation mixture transformed into *E. coli*. Plasmid DNA from transformants was isolated and the presence of the desired plasmid, designated ptrpR/hGH 107, confirmed. Messing et al., *Nucleic Acids Res.* 9, 309 (1981).

ptrpR/hGH107 was partially digested with EcoRI, blunt ended using Klenow, treated with PvuII to provide the lac promoter/trp repressor operon (the 530 b.p. fragment). Partial PvuII digestion of pFMB1 and isolation of vector fragment on 6 percent polyacrylamide provided the expression vector backbone containing the FMB coding sequence under control of the trp promoter. The ptrpR/hGH107 fragment was mixed with the pFMB1 PvuII digest and ligated with T4 ligase. The ligation mixture was then used to transform *E. coli* strain 294, and transformants used as a source of plasmid DNA. The resulting plasmid, pFMB/trpR was verified by miniscreen, and for orientation of the insert by Aval-PvuII digestion. Plasmid pFMB/trpR was digested with NdeI and BamHI. The fragment containing the trpR repressor was isolated. Plasmid pPA183'AX10 was digested with NdeI and BamHI. The main vector fragment was isolated. This vector fragment and trpR repressor fragment from pFMB trpR were ligated and the DNA mixture used to transform *E. coli* from which the plasmid pXAPA183'AX10trpR was isolated, as shown in Figure 8.



### 5. E. coli Expression Vectors for t-PA Mutants

Figure 8 depicts the pXAPPA183'Δx10trpR vector used to express t-PA and t-PA mutants in E. coli. As can be seen, the expression of the native t-PA structural gene is controlled by the trp promoter. Attention is directed to the XbaI, NarI and SacI restriction sites. Plasmid pXAPPA183' x10trpR was digested with NarI and XbaI. A 340 base pair fragment identified as fragment 1 in Figure 8 was isolated. A vector fragment identified as fragment 2 in Figure 8 was obtained by isolating the large fragment obtained by digestion of pXAPPA183'Δx10trpR with XbaI and SacI. Fragment 3 (900 bp) was obtained by digesting with NarI and SacI RF DNA of each of the mutant t-PA M13 clones, obtained by site specific mutagenesis. (Figure 3). Vectors expressing different mutant t-PAs were obtained by ligating fragments 1 and 2 with the respective fragments 3 and used to transform E. coli from which were isolated each of the E. coli mutant t-PA expression vectors:

20 pXAPPA18 3'Δx10trpR 1B8  
pXAPPA18 3'Δx10trpR 2C9  
pXAPPA18 3'Δx10trpR 4A10  
pXAPPA18 3'Δx10trpR 3A7  
pXAPPA18 3'Δx10trpR 4B3

These plasmids, as well as the wild type t-PA expression vector pXAPPA183'Δx10trpR were used to transform E. coli W3110fhuA<sup>-</sup>.

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E. coli W3110-fhuA<sup>-</sup> is a T1 phage resistant bacterium characterized by a deletion or inversion of DNA sequences associated with the fhuA gene.

Briefly, E. coli W3110 (ATCC 27325) is transduced with lambda bacteriophage containing the transposable element Tn10 which confers tetracycline resistance. Strains of Tn10 transduced W3110 are selected for resistance to phage infection. Phage resistant strains are pooled and infected with bacteriophage P1. The resulting lysate is used to transduce E. coli AT982 (Bukhari, et al., J. Bacteriology 105,844 (1971)). Strain AT 982

35

contains a Dap mutation located close to the *fhuA* gene. Accordingly, transduction of strain AT982 by the P1 lysate and selection of transductants which are tetracycline resistant and which regenerate the DAP function indicates that transposon *Tn10* is located within the *fhuA* gene. Strains which are tetracycline resistant and demonstrate regenerated DAP function are the source of DNA for bacteria phage P1 transduction of *E. coli* W3110. Transduced W3110 strains expressing tetracycline resistance and phage resistance are selected. These strains are then selected on the basis of resistance to phage infection and reversion to tetracycline sensitivity. Naloy, *et al.* J. Bacteriology 145, 1110(1981). The reversion to tetracycline sensitivity coupled with the retention of resistance to T1 phage infection indicates that DNA sequence associated with the *fhuA* gene have either been deleted or inverted irreversibly. Strains so constructed are designated *E. coli* W3110 *fhuA*<sup>-</sup>.

The phage containing the transposable element *Tn10* which was used to insert *Tn10* into W3110 was constructed as follows. The starting material was lambda cI857b 2210am29. This phage is known to those skilled in the art Kleckner, *J. Mol Biol.* 116, 125 (1977), and was constructed from three well known mutant of lambda by standard procedures. A lysate of this lambda phage was prepared on the amber suppressor *E. coli* C600 (ATCC No. 23724) which had been manipulated by procedures known to those skilled in the art to also carry the *Tn10* transposon. Kleckner, *et al.* J. Mol Biol. 116, 125(1977). This lysate was used to infect *E. coli* C600 (lambda C1857) which contains an amber suppressor and a lambda prophage carrying the cI857 genotype. Lysates of tetracycline resistant colonies were prepared by heat induction by growing the tetracycline resistant colonies first in broth at 32°C and thereafter at 42°C for 90 minutes. The lysate was then plated on *E. coli* C600 and replica plated. The plaques appearing on *E. coli* C600 were replica plated at 32°C on *E. coli* C600 and *E. coli* W3102 sup+ (lambda imm434) which contains the heteroimmune prophage lambda imm434. Kleckner, N. *et al.*, Genetics 90, 427(1978). Plaques appearing on the heteroimmune strain are plated onto tetracycline

plates. Plaques appearing on these plates are capable of transducing tetracycline resistance and are used in the above described method for generating *E. coli* W3110 *fnuA*<sup>+</sup>.

5 Native t-PA and mutant t-PA were obtained from 10 liter cultures of these cells transformed with the appropriate t-PA or mutant t-PA plasmid. Expression was induced by tryptophan deficient media.

6. Expression Vectors for t-PA Mutants in Mammalian Cells

10 The plasmid pPADHFR-6 (also designated pETPFR - see EPO application Publication No. 93619 supra) is depicted in Figure 9. The expression of the native t-PA structural gene is under the control of the early promoter for SV40 T-antigen. This promoter also controls the expression of the DHFR gene. Attention is directed to the BglII, BstXI and BstEII restriction sites. A vector fragment designated as fragment 1 in Figure 9 was obtained by isolating the large fragment generated by digestion of pPADHFR-6 with BglII and BstEII. The fragment designated as fragment 2 in Figure 9 was obtained by isolating the 400 base pair t-PA fragment obtained from the digestion of pPADHFR-6 with BglII and BstXI. A 1,141 base pair t-PA fragment containing the desired mutations and corresponding to fragment 3 in Figure 9 was obtained by digesting RF DNA from each of the mutant t-PA clones with BstXI and BstEII. Fragments 1 and 2 were ligated with each fragment 3. The DNA mixtures were used to transform *E. coli*. From each of the transformants, the respective eukaryotic expression vectors were obtained:

30 pPADHFR-6 1B8  
pPADHFR-6 2C9  
pPADHFR-6 4A10  
pPADHFR-6 3A7  
pPADHFR-6 4B3

These plasmids, as well as the non-mutated t-PA expression vector pPADHFR-6, were used to transfect DHFR deficient CHO cells as disclosed supra. (Graham et al., Virology 52, 456 (1973); see also EPO Publ. No. 093619) Native and mutant t-PA expression was amplified by

exposing cultures to increasing concentrations of methotrexate.

For example, plasmids pPADHFR-6 2C9 and pPADHFR-6 1B8 were used to transfect DHFR deficient CHO cells [Urlab & Chasin (PNAS 77, 4216 (1980))] using the calcium phosphate precipitation method of Graham et al., Virology 52, 456 (1973).

In each case, the colonies that arose in selective medium (medium lacking hypoxanthine, glycine, and thymidine (-HGT) were pooled and grown further in -HGT medium. These cells were plated at  $2 \times 10^5$  cells per 100 mm plate in 250 nM methotrexate (MTX) to select for amplification of plasmid sequences. Five clones that grew in 250 nM MTX were extracted from the plate and all were found to be secreting t-PA into the medium. These clones were used for further study.

#### E. Assay Methods

##### 1. Mutant t-PA and t-PA Purification

The various t-PAs expressed in mammalian cells as described above were secreted into the cell culture medium. The medium containing such t-PAs was used directly in various assays to be described hereafter or was subjected to one or more of the following purification steps to increase the purity of t-PA or mutant t-PA prior to such assays.

Media from CHO cells containing mutant t-PA was batch extracted with chelating Sepharose (Pharmacia) (10-20 mL resin/L media) activated with zinc chloride as described by Rijken, et al., Biochim. & Biophys. Acta 580, 140 (1979) and collected on a filter. The resin was poured into a column, washed with a buffer containing 0.02 M sodium phosphate, pH 8.0, 0.25 M NaCl, 0.01 percent TWEEN 80 and 10 mg/liter aprotinin. The t-PA was eluted with the same buffer containing 50 mM imidazole. The t-PA pool was dialyzed into 0.02 M sodium phosphate, pH 8, 0.25 M NaCl and 0.01 percent TWEEN 80 and loaded onto a lysine Sepharose resin, Radcliffe et al., Arch. Biochem. Biophys. 189, 185 (1978) and Allen, et al., Thrombosis Hemostasis 45, 43 (1981), or

benzamidine Sepharose resin, Bykowska, *et al.*, Biochim. & Biophys. Acta, 703, 113 (1982). The zinc chelate resin was washed briefly with 0.02 M sodium phosphate, pH 8, 1 M NaCl and 0.01 percent TWEEN 80 and t-PA or mutant t-PA eluted with the same buffer containing 0.5 M arginine. The benzamidine sepharose was washed with the dialysis buffer and eluted with the dialysis buffer containing 1M guanidine. The resulting proteins were greater than 90% pure as analysed by SDS-PAGE. In addition to the use of the foregoing purification techniques, immobilized monoclonal antibodies may be used (See Nielsen *et al.*, EMBO J. 2, 115 (1983).

## 2. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples of media containing t-PA protein or the t-PA mutant proteins were concentrated by vacuum and diluted into sodium dodecyl sulfate (SDS) sample buffer. Where indicated, 10mM dithiothreitol (DTT) was added to reduce the protein disulfides. Discontinuous SDS electrophoresis using 10% or 7 to 17% polyacrylamide resolving gels was performed according to the procedure of Laemmli. [Laemmli, Nature 227, 680 (1970)]. For analysis of plasma samples, 4% to 10% SDS polyacrylamide gradient resolving gels were used with the buffer system of Laemmli. Estimated molecular weights (Mr) from SDS-PAGE analysis were obtained by comparison to the mobility of protein of known molecular weight.

## 3. Bubble Release Clot Lysis

Recombinant (non-mutant) t-PA and mutant t-PAs hereof (mutant t-PA) were assayed for their ability to solubilize fibrin clots by the bubble release clot lysis assay.

Briefly, thrombin (Sigma Chemical Co.) was dissolved in distilled water to approximately 1000 units/ml. This stock solution was diluted 1:30 with assay buffer which contained 0.06M monobasic sodium phosphate, 0.06 M dibasic sodium phosphate, 200 mg/liter sodium azide and 0.01% TWEEN 80. A series of test tubes containing 0.5 ml of diluted thrombin (30-40 units/ml) and 0.5 ml of either various

concentrations of t-PA (16 ng/ml to  $1 \times 10^6$  ng/ml); appropriate controls or unknown sample in appropriate dilutions were prepared. A second series of test tubes containing 20  $\mu$ l plasminogen (1.0 mg/ml), and 1.0 ml of fibrinogen (1 mg/ml) and 10  $\mu$ l of hollow glass microspheres greater than 45 mesh (3M Company) was also prepared.

The above reagents and test tubes were kept on ice until the final step of the assay. 200  $\mu$ l of either the thrombin-t-PA or thrombin-mutant t-PA solutions were added sequentially to a test tube containing the plasminogen, fibrinogen, and microspheres, vortexed for 15 seconds and placed in a 37°C water bath. Clots formed in each tube within 30 seconds. The time between t-PA addition and the endpoint of the reaction was measured. The endpoint was defined as the time when the microspheres in the assay had risen to the surface.

The amount of thrombolytic activity of a particular sample was determined by reference to a standard t-PA curve. Specific activity was calculated based on the amount of t-PA or mutant t-PA present as determined by radioimmunoassay.

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#### 4. In Vitro Clot Lysis Assay

Recombinant t-PA and mutant t-PA were also assayed in an in vitro clot lysis system.

Briefly, human blood was collected with 3.13% sodium citrate as anticoagulant and the cellular fraction removed by centrifugation. 50  $\mu$ l of 0.5M  $\text{CaCl}_2$  25  $\mu$ l bovine-thrombin (100 units/ml) and 10  $\mu$ l of human  $^{125}\text{I}$ -fibrinogen (100,000 cpm/10 $\mu$ l) was added to each ml of plasma. This plasma mix was aspirated into silicon tubing with an inside diameter of 4mm and incubated at 37°C for 1 hour. Segments (1 cm) of the tubing were cut and the clot removed. The clots were placed in buffer consisting of 0.3M NaCl, 0.02M sodium citrate, pH 5, and 0.01% TWEEN 80. The clots were rinsed four times in one hour with fresh buffer. The amount of radioactivity in the last rinse did not exceed about 10% of the amount of radioactivity in the clot. Each clot was

placed in 2.5 ml of plasma. A 250  $\mu$ l sample of plasma was taken as a zero point. A sample of t-PA or mutant t-PA was added in a volume of 100  $\mu$ l. Samples (250  $\mu$ l) were taken at 1, 2, 3 and 4 hours and the radioactivity contained therein determined. Standards containing 5, 10, 20 and 40 units of t-PA activity per ml were run in parallel. The percent lysis was calculated after correction for volume changes after each sample.

#### 5. Chromogenic Assays

10 S-2288: t-PA may be measured directly using the Kabi synthetic tripeptide chromogenic substrate, S-2288 (Helena Laboratories, Beaumont, Texas). For this assay, t-PA and 1 mM S-2288 (final concentration) in 0.05 M Tris, pH 7.4 containing 0.012 M NaCl and 0.01 percent TWEEN 80 were incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 50  $\mu$ l of glacial acetic acid to 0.5 ml reaction mixture. The activity was calculated from the absorbance at 405 nm using the following equation, standardized by the manufacturer:

$$20 \quad \text{Activity in 0.5 ml reaction mixture (IU, international units)} = \frac{\Delta OD \times 793.65 \times \frac{\text{IU-min}}{\text{OD}}}{\text{time of incubation}}$$

25 S-2251: Plasminogen activation by t-PA was measured using the Kabi specific tripeptide chromogenic substrate specific for plasmin, S-2251 (Helena Laboratories). An aliquot of the sample was mixed with 0.10 ml of 0.7 mg/ml plasminogen (0.05 M Tris, pH 7.4 containing 0.012 M NaCl) plus 0.02 ml of human fibrinogen 20 mgs/ml (0.05M Tris HCl, pH 7.4, containing 0.012 M NaCl) and the volume adjusted to 0.15 ml. The mixture was incubated at 37°C for 10 minutes, 0.35 ml of S2251 (1.0 mM solution in above buffer) was added, and the reaction continued for 5 or 10 minutes at 37°C. Glacial acetic acid (50  $\mu$ l) was added to terminate the reaction and the absorbance at 405 nm was measured. Quantitation of the amount of activity was obtained by comparison to the results obtained using a recombinant native t-PA sample which had been standardized using the S-2288 assay. This was

necessary initially because the absorbance at 405 nm varied from day to day as the plasminogen aged and also changed if different preparations of plasminogen and fibrinogen were used. This variability was ultimately reduced by careful preparation of large amounts of human plasminogen (glu-plasminogen) with subsequent lyophilization of aliquots of the material. The aliquots were stored at -20°C. Prior to use, the redissolved plasminogen preparations were stored at 0°C for not more than 4 hours. Stimulation of t-PA activity by fibrinogen was measured by comparing the activity of solutions containing high concentrations of fibrinogen to similar reaction mixtures in which fibrinogen had been omitted. Due to the insolubility of fibrin, fibrinogen was used in this assay. The stimulation by high concentrations of fibrinogen appears to mimic the stimulation that would be expected by the insoluble fibrin.

#### 6. In Vivo Inhibitor-Complex Assay

Recombinant t-PA and mutated t-PA were assayed in vitro to determine their reactivity with naturally occurring inhibitors of t-PA activity. Generally, t-PA and mutant t-PA were iodinated with  $^{125}\text{I}$  by using Iodobeads (Pierce Chemical Co.) resulting in t-PA or mutant t-PA having specific radioactivities approximately  $2 \times 10^6$  cpm/ $\mu\text{g}$ . For in vitro complex formation, the radiolabeled t-PA (1  $\mu\text{g}$ ) was added to freshly drawn citrated human whole blood (500  $\mu\text{l}$ ). The samples were incubated at room temperature and the reaction stopped by dilution of an aliquot into 2% SDS. Samples were analyzed in 4 to 10% polyacrylamide gradient SDS-PAGE. Complexes were detected by autoradiography.

#### 7. Fibrin Binding Assay

The method for fibrin binding is a modification of the method described by Rijken *et al.*, J. Biol. Chem. **257**, 2920 (1982). The t-PA sample to be tested (500 ng) is added to a solution containing 0.05 M Tris, pH 7.4, 0.12 M NaCl, 0.01% TWEEN 80, 1 mg/ml human serum albumin, and various concentrations of plasminogen free fibrinogen (0, 0.1, 0.5 and 1.0 mg/ml). The final volume of the reaction mixture is 1 ml. The



sample is incubated at 37°C for five minutes, followed by the addition of 1 unit of thrombin. The samples are incubated for one hour at 37°C. The clot is removed using a glass rod, and the amount of t-PA remaining unbound in the supernatant is determined. The data is plotted as percent t-PA bound versus the fibrinogen concentration (Figure 11).

#### 8. In Vivo Clot Lysis

The in vivo clot lysis model of Collen et al., J. Clin. Invest. 71, 368 (1983), was used. Male New Zealand white rabbits between 2.5 and 3 kg were anesthetized with ketamine, the jugular vein was catheterized and small communicating vessels in the region were ligated. Approximately 2 cm of the jugular was isolated with reversible ligatures, a thread was passed from the proximal to the distal end of the segment, the segment was flushed with a saline thrombin solution and filled with fresh rabbit blood which contained <sup>125</sup>I human fibrinogen. After 30 minutes blood flow was resumed across the clot. The t-PA i.v. infusion was started with an initial bolus of 10% of the total dose. The infusion was delivered over 4 hours. Thirty minutes after the end of the infusion the clot was harvested and counted. The recovery of radioactivity was used as a quality control; blood samples, urine, swabs and syringes were counted to assure that the estimate of the amount of radioactivity present in the initial clot was accurate.

#### 25 F. Assay Results

t-PA mutants with the following sequences at the two-chain activation site, residues 270 through 279, have been expressed in both E. coli and Chinese Hamster Ovary cells (CHO cells):

	275	279
30 native	-Arg-Ile-Lys-Gly-Gly-	(RIKGG)
1B8	-Gly-Ile-Lys-Gly-Gly-	(GIKGG)
2C9	-Glu-Ile-Lys-Gly-Gly-	(EIKGG)

#### 1. Western Blots and Zymography

The EIKGG & GIKGG mutants expressed in CHO cells were analyzed by Western blots derived from reduced and non-reduced SDS-PAGE

gels. Native single-chain t-PA shows up as two bands having molecular weights of 52,000 and 50,000 daltons due to a difference in the extent of glycosylation. The EIKGG mutant from a non-reduced SDS-PAGE showed one major immunoreactive band at a molecular weight of approximately 50,000 daltons. The Western blot of the mutant GIKGG from a non-reduced SDS-PAGE, however, showed a molecular weight of 55,000 daltons. The difference in apparent molecular weight of the GIKGG mutant as compared to native t-PA may indicate a slightly different conformation or carbohydrate structure compared to native t-PA. Cleavage of the protein at arg 275 can be detected by a lower molecular weight of t-PA when analyzed following reduction (thereby separating the protease and Kringle chains). Zymographs of the reduced SDS-PAGE gels showed that plasminogen activator activity in these samples was at the molecular weight of the immunoreactive band of the single-chain form of t-PA (approximately 60,000). The two-chain form of t-PA has an electrophoretic mobility consistent with a molecular weight of approximately 30,000 daltons. This procedure demonstrated that single-chain forms of the mutant t-PA proteins were present in the media from transformed cells.

#### S-2251 Assay

Analysis of the native and a mutant EIKGG t-PA by the S-2251 assay is shown in Table I. These values were obtained prior to the use of glu-plasminogen in the assay in order to decrease assay variability.

25 The naturally occurring t-PA sequence RIKGG was assigned an arbitrary specific activity in the presence of fibrinogen on the basis of the S2288 assay. This standard t-PA was assayed with each of the EIKGG t-PA mutants to normalize results.

30 As can be seen the EIKGG t-PA mutant, regardless of the degree of purification, has a specific activity in the S2251 plus fibrinogen assay greater than that for the recombinant t-PA.

TABLE I

	<u>Mutation</u>	<u>Mutant</u>	<u>S-2251 + Fibrinogen</u>	<u>S-2251 - Fibrinogen</u>	<u>Fibrinogen Stimulation</u>
5	RIKGG <sup>1</sup>	native	(250,000) <sup>4</sup>	25,000	10.0
	EIKGG <sup>1</sup>	2C9	1,000,000	3,400	290.0
	EIKGG <sup>2</sup>	2C9	420,000	3,100	134.0
10	EIKGG <sup>3</sup>	2C9	520,000	7,000	74.0

<sup>1</sup> purified using zinc chelate lysine-agarose

<sup>2</sup> purified using zinc chelate and benzamidine agarose

<sup>3</sup> assayed with no purification

15

<sup>4</sup> assigned activity

20 The data in Table IA were obtained using high quality, lyophilized, glu-plasminogen. With a more reproducible assay, the EIKGG mutant was found to be equal in activity in the S-2251 assay in the presence of fibrinogen. In the absence of fibrinogen, the mutant was still less active than native (Tables I and IA), demonstrating a greater specificity.

25

TABLE IA

	<u>Mutation</u>	<u>Mutant</u>	<u>S-2251 + Fibrinogen</u>	<u>S-2251 - Fibrinogen</u>	<u>Fibrinogen Stimulation</u>
	RIKGG <sup>1</sup>	native	(250,000) <sup>2</sup>	17,600	14
30	EIKGG <sup>1</sup>	2C9	248,000	500	500

<sup>1</sup> purified using zinc chelate lysine-agarose

<sup>2</sup> assigned activity

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## 2. Bubble Release Clot Lysis and In Vitro Clot Lysis Assay

The bubble release clot lysis assay was used to determine the specific activity of recombinant t-PA and the purified EIKGG t-PA mutant. The activity of each of these t-PAs was determined by the procedures described above. The concentration of t-PA and EIKGG mutant t-PA was determined by radioimmunoassay. The results of this assay including specific activity are shown in Table II.

TABLE II

<u>Sample</u>	<u>I.D.</u>	<u>U/ml</u> <u>Activity</u>	<u>Protein</u> <u>Conc. mg/ml</u>	<u>Specific</u> <u>Activity</u>
1	EIKGG*	8440	0.088	95,909
2	EIKGG*	7698	0.088	87,477
3	t-PA**	5640	0.088	64,090

1) Frozen - thawed once

2) Frozen - thawed four times

\* purified using zinc-chelate and benzamidine-agarose

\*\* purified using zinc-chelate and lysine-agarose

The bubble release clot lysis assay demonstrates that a one-chain mutant of t-PA, specifically the EIKGG mutant t-PA, has a specific activity 50% greater than recombinant t-PA. As can be seen repeated freezing and thawing resulted in a slight decrease in the specific activity of the EIKGG t-PA mutant. However, the mutant t-PA still maintained a specific activity greater than that of the recombinant t-PA.

## 3. In Vivo Inhibitor-Complex Assay

The inactivation of proteases by plasma protease inhibitors is a well-studied mechanism for inactivating serum proteases. The resulting complexes are stable to denaturation and can be assessed by

electrophoresis on SDS-PAGE. In this procedure, radiolabeled t-PA is added to plasma or whole blood and the sample incubated at 37°C. The sample is subjected to SDS-PAGE followed by autoradiography. The detection of radiolabel at positions of Mr greater than free t-PA is an indication of the amount of t-PA protease inhibitor complex which has been formed. When analyzed in rat blood, t-PA was found to slowly form complexes with Mr greater than 200,000. After several hours of incubation, greater than 70% of the radiolabel could be detected in such complexes. In contrast, the mutated t-PA did not form these complexes; the bulk of the radiolabel detected by autoradiography remained at the position of free, uninactivated enzyme. When a similar analysis was performed in human blood, (Figure 10) t-PA also formed such complexes, but in addition formed complexes of Mr between 100,000 and 200,000. As with the rat blood, the mutant t-PA formed markedly less inhibitor complexes with Mr greater than 200,000. The protease inhibitor complexes with Mr values between 100,000 and 200,000 were still present. These results indicate that the mutant is not inactivated by the proteinase inhibitor(s) which form complexes with Mr values greater than 200,000. Species differences are noted in the reactivity of both t-PA and the mutated t-PA in the formation of complexes between 100,000 and 200,000.

#### 4. Fibrin Binding

It has previously been reported that one-chain and two-chain forms of t-PA have approximately equal affinity for fibrin (Rijken *et al.*, J. Biol. Chem. 257, 2920 (1982). In the assay described herein, in contrast, a markedly higher affinity for fibrin observed for the one-chain form of t-PA as compared to the two-chain form (Figure 11).

#### 5. In Vivo Clot Lysis

Figure 12 shows the relative dose response curves for t-PA (o) and the EIK mutant (o). The data are presented as the Mean  $\pm$  SEM with 5 rabbits in each group. The distance between the two curves at the 50% lysis point was measured and the potency of the EIK form of t-PA was estimated to be 2.4 times greater than the non-mutated form

(RIK). A statistically significant difference was achieved at the 0.25 mg/kg dose ( $p < 0.01$ ).

G. Conclusion

5 The above results demonstrate that mutation at residue 275 of t-PA may be more efficacious than the natural form for two separate reasons:

1. Increased specificity: Assays of t-PA function indicate a more active/specific protein.

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2. Decreased in vivo plasma inhibitor binding: in vivo inhibition of such mutants indicate a decrease in inactivation by certain protease inhibitors. This should allow for the circulation of the active uncomplexed form of t-PA thereby allowing for increased functional t-PA to dissolve a clot.

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The scientific literature is contradictory on the enzymatic properties of the one-chain form of t-PA. In order to better understand the function of t-PA one can look to homologous proteins. Extensive investigations have been performed in the serine proteases trypsin and chymotrypsin. The t-PA protease domain is very similar to these proteins and is expected to function in a similar manner. Based on the mechanism of function determined for trypsin and chymotrypsin, preventing cleavage at arginine 275 of t-PA would be expected to affect only the functional characteristics of the protease domain. The increased fibrin affinity of the mutants is therefore surprising.

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Regardless of the mechanism(s) involved (increased specificity, lack of protease inhibitor binding, increased affinity for fibrin, or combination of these), when one mutant was tested for its ability to lyse a blocked vein in vivo, it was found to be approximately 2.5 times more active than the t-PA of natural sequence. As discussed previously, the one-chain form of t-PA has been shown to be converted to the two-chain form at the site of a clot. Such a conversion would

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destroy any advantage associated with the one-chain form. Only a mutated form of t-PA is capable of being converted to the two-chain form by physiologic proteases will be able to preserve its advantages once at the site of a clot.

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Having described the preferred embodiment of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiment, and that such modifications are intended to be within the scope of the present invention.

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CLAIMS:

1. A human tissue plasminogen activator (t-PA) mutant produced by recombinant host cells, and resistant to  
5 specific enzymatic cleavage.
2. A mutant according to Claim 1 essentially free of two-chain form.
- 10 3. A single-chain mutant according to Claim 1.
4. A single-chain mutant according to Claim 3 stabilized in single-chain form by site directed mutagenesis at a two-chain cleavage site.
- 15 5. A mutant according to Claim 4 in which position 275 is occupied by an amino acid other than arginine.
6. A mutant according to Claim 5 in which said amino  
20 acid is glycine or glutamic acid.
7. A mutant according to Claim 4 wherein said two-chain activation site is disposed in the range of residues 270 through 279 of t-PA and said site directed mutagenesis is  
25 accomplished by substitution of at least one amino acid residue within said two-chain activation site.
8. A mutant according to Claim 7 wherein said substitution is accomplished by replacing isoleucine at residue 276.
- 30 9. A mutant according to Claim 7 wherein said substitution is accomplished by replacing arginine at residue 275.
10. A single-chain tissue plasminogen activator mutant  
35 produced by recombinant host cells, having decreased



affinity for protease inhibitors.

11. A single-chain tissue plasminogen activator mutant produced by recombinant host cells having a higher specific  
5 activity for plasminogen conversion than non-mutated tissue plasminogen activator.

12. A mutant according to Claim 1 stabilized against enzymatic hydrolysis at a trypsin-like cleavage site, said  
10 cleavage site being characterized by arginine or lysine, wherein one or more amino acids of said cleavage site is substituted by a different amino acid.

13. A DNA sequence encoding the mutants according to any  
15 one of the preceding claims.

14. A replicable expression vector capable, in a transformant host cell, of expressing the DNA sequence of Claim  
13.

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15. A microorganism transformed with the vector of Claim  
14.

16. A cell culture transformed with the vector of Claim  
25 14.

17. A mammalian cell culture transformed with the vector of Claim 14.

30 18. The cell culture of Claim 17 obtained by transforming a Chinese Hamster Ovary cell line.

19. A composition comprising a therapeutically effective amount of plasminogen activator mutant of Claims 1 to 12 in  
35 admixture with a pharmaceutically acceptable carrier.

20. A human tissue plasminogen activator mutant of any one of Claims 1 to 12 for use in treating vascular disease or conditions in a subject.

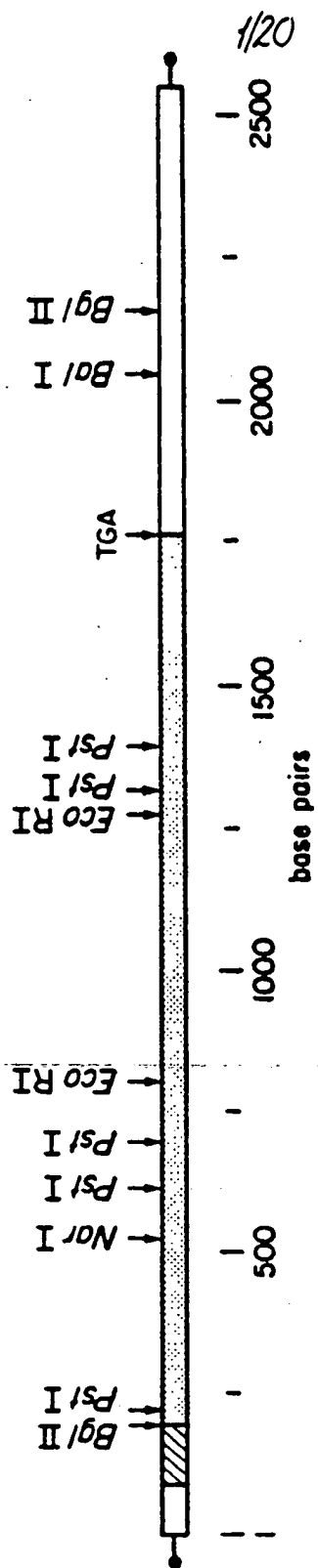


Fig. 1.

Fig. 2A.

GTTCTGAGCACAGGGCTGGAGAGAAACCTCTGCGAGGAAAGGAGGCAAGCCGTGA

ATTTAAGGGACGCTGTGAAGCAATGC      -35      met asp ala met lys arg gly leu  
ATG GAT GCA ATG AAG AGA GGG CTC      -30

cys cys val leu leu leu cys gly ala val phe val ser pro ser  
TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC      -20

gln glu ile his ala arg phe arg arg gly ala arg ser tyr gln  
CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA TCT TAC CAA      -10      1

VAL ILE CYS ARG ASP GLU LYS THR GLN MET ILE TYR GLN GLN HIS  
GTG ATC TGC AGA GAT GAA AAA ACG CAG ATG ATA TAC CAG CAA CAT      10

GLN SER TRP LEU ARG PRO VAL LEU ARG SER ASN ARG VAL GLU TYR  
CAG TCA TGG CTG CGC CCT GTG CTC AGA AGC AAC CCG GTG GAA TAT      20      30

CYS TRP CYS ASN SER GLY ARG ALA GLN CYS HIS SER VAL PRO VAL  
TGC TGG TGC AAC AGT GGC AGG GCA CAG TGC CAC TCA GTG CCT GTC      40

LYS SER CYS SER GLU PRO ARG CYS PHE ASN GLY GLY THR CYS GLN  
AAA AGT TGC AGC GAG CCA AGG TGT TTC AAC GGG GGC ACC TGC CAG      50      60

3/20

Fig. 2B.

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70
GLN ALA LEU TYR PHE SER ASP PHE VAL CYS GLN CYS PRO GLU GLY
CAG GCC CTG TAC TTC TCA GAT TTC GTG TGC CAG TGC CCC GAA GGA

80
PHE ALA GLY LYS CYS CYS GLU ILE ASP THR ARG ALA THR CYS TYR
TTT GCT GGG AAG TGC TGT GAA ATA GAT ACC AGG GCC ACG TGC TAC

90
100
GLU ASP GLN GLY ILE SER TYR ARG GLY THR TRP SER THR ALA GLU
GAG GAC CAG GGC ATC AGC TAC AGG GGC ACG TGG AGC ACA GCG GAG

110
SER GLY ALA GLU CYS THR ASN TRP ASN SER SER ALA LEU ALA GLN
AGT GGC GCC GAG TGC ACC AAC TGG AAC AGC AGC GCG TTG GCC CAG

120
130
LYS PRO TYR SER GLY ARG ARG PRO ASP ALA ILE ARG LEU GLY LEU
AAG CCC TAC AGC GGC CGG AGG CCA GAC GCC ATC AGG CTG GGC CTG

140
GLY ASN HIS ASN TYR CYS ARG ASN PRO ASP ARG ASP SER LYS PRO
GGG AAC CAC AAC TAC TGC AGA AAC CCA GAT CGA GAC TCA AAG CCC

150
160
TRP CYS TYR VAL PHE LYS ALA GLY LYS TYR SER SER GLU PHE CYS
TGG TGC TAC GTC TTT AAG GCG GGG AAG TAC ACG TCA GAG TTC TGC

170
SER THR PRO ALA CYS SER GLU GLY ASN SER ASP CYS TYR PHE GLY
AGC ACC CCT GCC TGC TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG

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Fig. 2C.

190  
 ASN GLY SER ALA TYR ARG GLY THR HIS SER LEU THR GLU SER GLY  
 AAT GGG TCA GCC TAC CGT GGC ACG CAC AGC CTC ACC GAG TCG GGT

200  
 ALA SER CYS LEU PRO TRP ASN SER MET ILE LEU ILE GLY LYS VAL  
 GCC TCC TGC CTC CCG TGG AAT TCC ATG ATC CTG ATA GGC AAG GTT

210  
 220  
 TYR THR ALA GLN ASN PRO SER ALA GLN ALA LEU GLY LEU GLY LYS  
 TAC ACA GCA CAG AAC CCC AGT GCC CAG GCA CTG GGC CTG GGC AAA

230  
 HIS ASN TYR CYS ARG ASN PRO ASP GLY ASP ALA LYS PRO TRP CYS  
 CAT AAT TAC TGC CGG AAT CCT GAT GGG GAT GCC AAG CCC TGG TGC

240  
 250  
 HIS VAL LEU LYS ASN ARG ARG LEU THR TRP GLU TYR CYS ASP VAL  
 CAC GTG CTG AAG AAC CGC AGG CTG ACG TGG GAG TAC TGT GAT GTG

260  
 PRO SER CYS SER THR CYS GLY LEU ARG GLN TYR SER GLN PRO GLN  
 CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG TAC AGC CAG CCT CAG

270  
 280  
 PHE ARG ILE LYS GLY GGA GGG CTC TTC GCC GAC ATC GCC TCC CAC CCC  
 TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC ATC GCC TCC CAC CCC

290  
 300  
 TRP GLN ALA ALA ILE PHE ALA LYS HIS ARG ARG SER PRO GLY GLU  
 TGG CAG GCT GCC ATC TTT GCC AAG CAC AGG AGG TCG CCC GGA GAG

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Fig. 2D.

ARG PHE LEU CYS GLY GLY ILE LEU ILE SER SER CYS TRP ILE LEU  
 CGG TTC CTG TGC GGC GGC ATA CTC ATC AGC TCC TGC TGG ATT CTC  
 310  
 SER ALA ALA HIS CYS PHE GLN GLU ARG PHE PRO PRO HIS HIS LEU  
 TCT GCC GCC CAC TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC CTG  
 320  
 THR VAL ILE LEU GLY ARG THR TYR ARG VAL VAL PRO GLY GLU GLU  
 ACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG GAG  
 340  
 GLU GLN LYS PHE GLU VAL GLU LYS TYR ILE VAL HIS LYS GLU PHE  
 GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC  
 350  
 ASP ASP ASP THR TYR ASP ASN ASP ILE ALA LEU LEU GLN LEU LYS  
 GAT GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG CAG CTG AAA  
 370  
 SER ASP SER SER ARG CYS ALA GLN GLU SER SER VAL VAL ARG THR  
 TCG GAT TCG TCC CGC TGT GCC CAG GAG AGC AGC GTG GTG CGC ACT  
 380  
 VAL CYS LEU PRO PRO ALA ASP LEU GLN LEU PRO ASP TRP THR GLU  
 GTG TGC CTT CCC CCG GCG GAC CTG CAG CTG CCG GAC TGG ACG GAG  
 400  
 CYS GLU LEU SER GLY TYR GLY LYS HIS GLU ALA LEU SER PRO PHE  
 TGT GAG CTC TCC GGC TAC GGC AAG CAT GAG GCC TTG TCT CCT TTC  
 410  
 420

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Fig. 2E.

TYR SER GLU ARG LEU LYS GLU ALA HIS VAL ARG LEU TYR PRO SER  
 TAT TCG GAG CGG CTG AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC  
 430  
 SER ARG CYS THR SER GLN HIS LEU LEU ASN ARG THR VAL THR ASP  
 AGC CGC TGC ACA TCA CAA CAT TTA CTT AAC AGA ACA GTC ACC GAC  
 440  
 ASN MET LEU CYS ALA GLY ASP THR ARG SER GLY GLY PRO GLN ALA  
 AAC ATG CTG TGT GCT GGA GAC ACT CGG AGC GGC GGC CCC CAG GCA  
 450  
 ASN LEU HIS ASP ALA CYS GLN GLY ASP SER GLY GLY PRO LEU VAL  
 AAC TTG CAC GAC GAC GCC TGC CAG GGC GAT TCG GGA GGC CCC CTG GTG  
 460  
 CYS LEU ASN ASP GLY ARG MET THR LEU VAL GLY ILE ILE SER TRP  
 TGT CTG AAC GAT GGC CGC ATG ACT TTG GTG GGC ATC ATC AGC TGG  
 470  
 GLY LEU GLY CYS GLY GLN LYS ASP VAL PRO GLY VAL TYR THR LYS  
 GGC CTG GGC TGT GGA CAG AAG GAT GTC CCG GGT GTG TAC ACA AAG  
 480  
 VAL THR ASN TYR LEU ASP TRP ILE ARG ASP ASN MET ARG PRO OP  
 GTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC ATG CGA CCG TGA  
 490  
 500  
 510  
 520  
 527

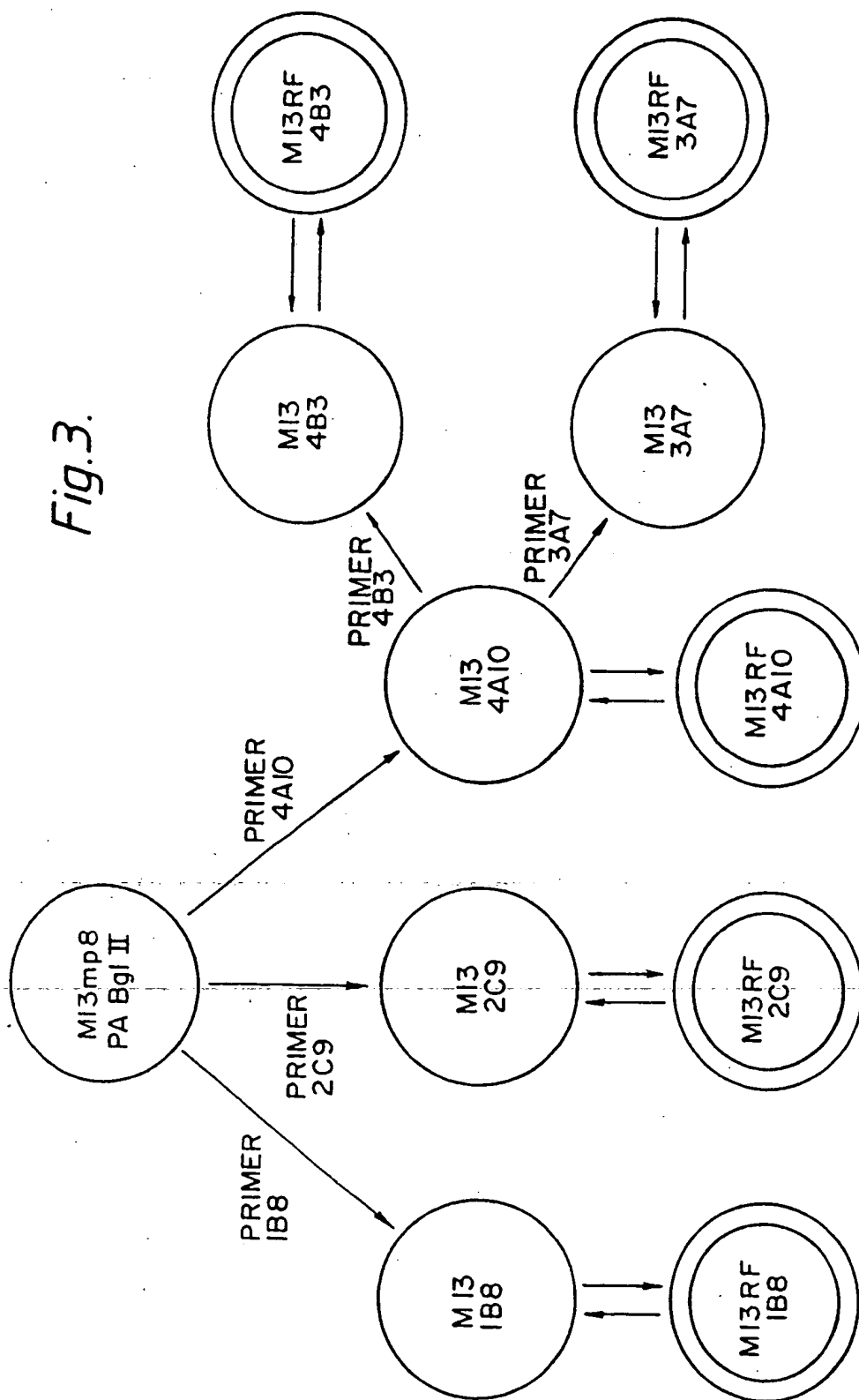


Fig. 2F.

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ACGAGACCCCTACAGGGAGAGGAGTGCTTTTCCAGATACTTCCCATTTTGGAAAGT  
TTTCAGGACTTGGTCTGATTTCAGGATACTCTGTGAGATGGGAAGACATGAATGCACACT  
AGCCTCTCCAGGAATGCCTCCTCCCTGGGCAGAAAGTGCCCATGCCACCCCTGTTTTCGCTA  
AAGCCCAACCTCCTGACCTGTCAACCGTGAGCAGCTTTGGAAACAGGACCCACAAAAATGAA  
AGCATGTCTCAATAGTAAAGAAACAAGAGATCTTTCAGGAAAGACGGATTGCATTAGAA  
ATAGACAGTATATTTATAGTCACAAGGGCCCGCAGCAGGGCTCAAAGTTGGGGCAGGCTGGC  
TGGCCCGTCATGTTCTCAAAGCGCCCTTGACGTCAAGTCTCCTTCCCCCTTCCCCCACT  
CCCTGGCTCTCAGAAGGTATTCTTTTGAGTACAGTGTGTAAAGTGTAAATCCTTTTTCT  
TTATAAAGTTTAGAGTAGCATGAGAGAATTGTATCATTTGAACAACCTAGGCTTCAGCATA  
TTTATAGCGATCCATCGTTAGTTTTTACITTCGGTTGCCACAACCCCTGTTTTATACCGTA  
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Fig. 3.



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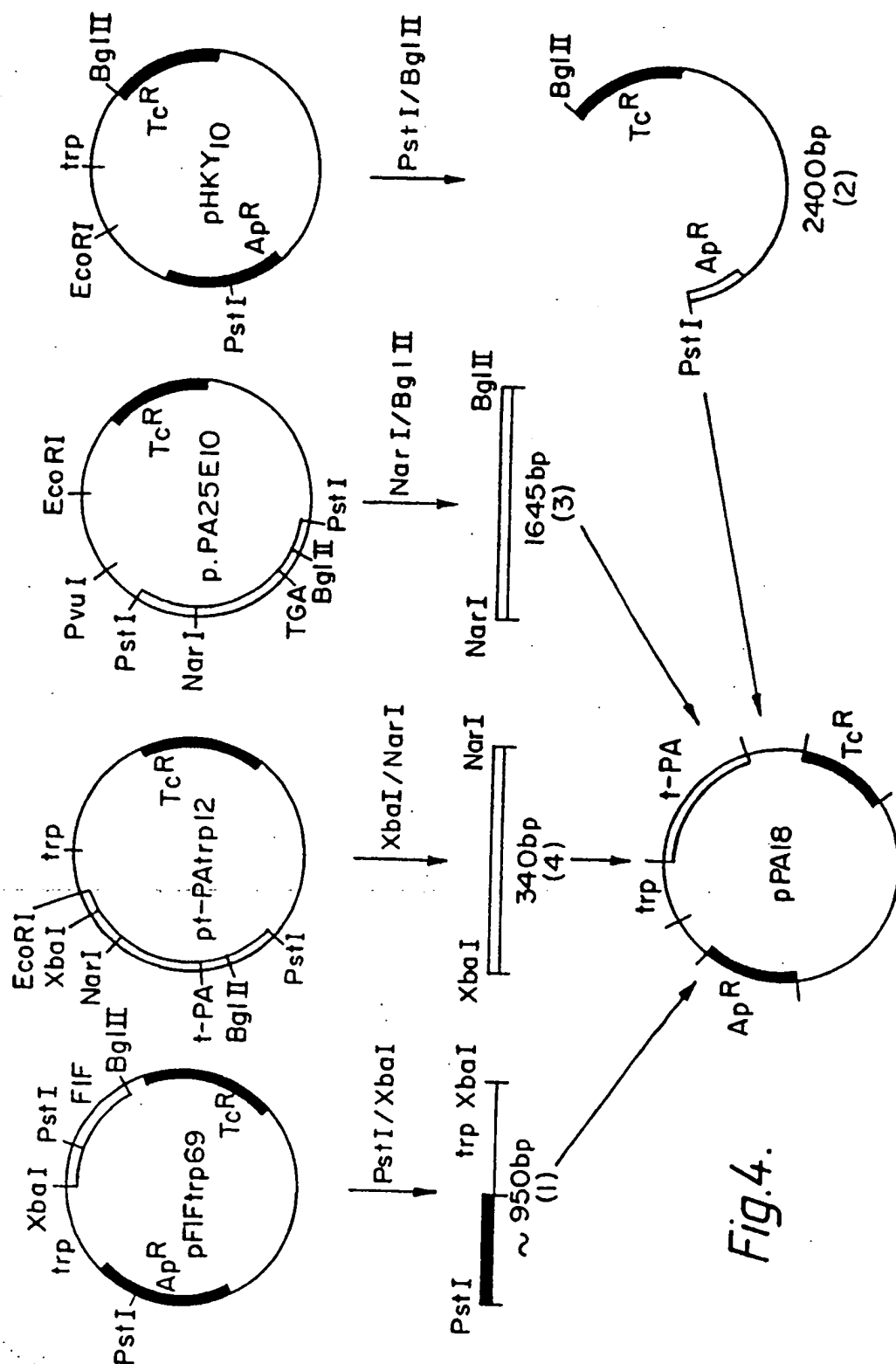
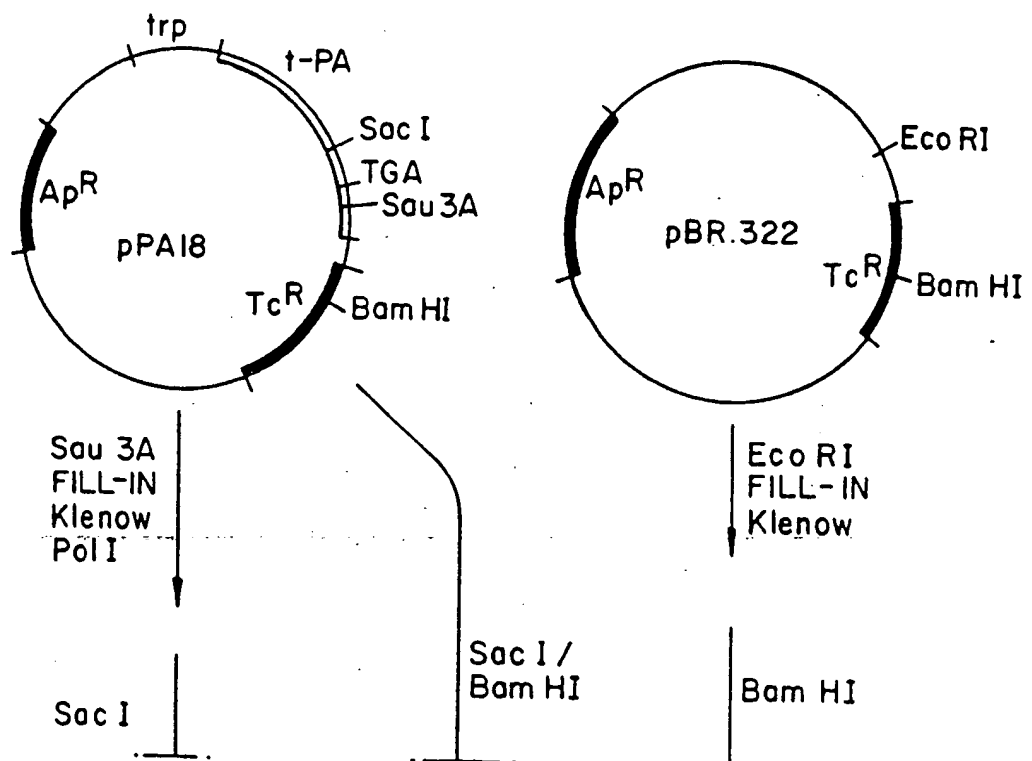


Fig.4.

Fig.5.



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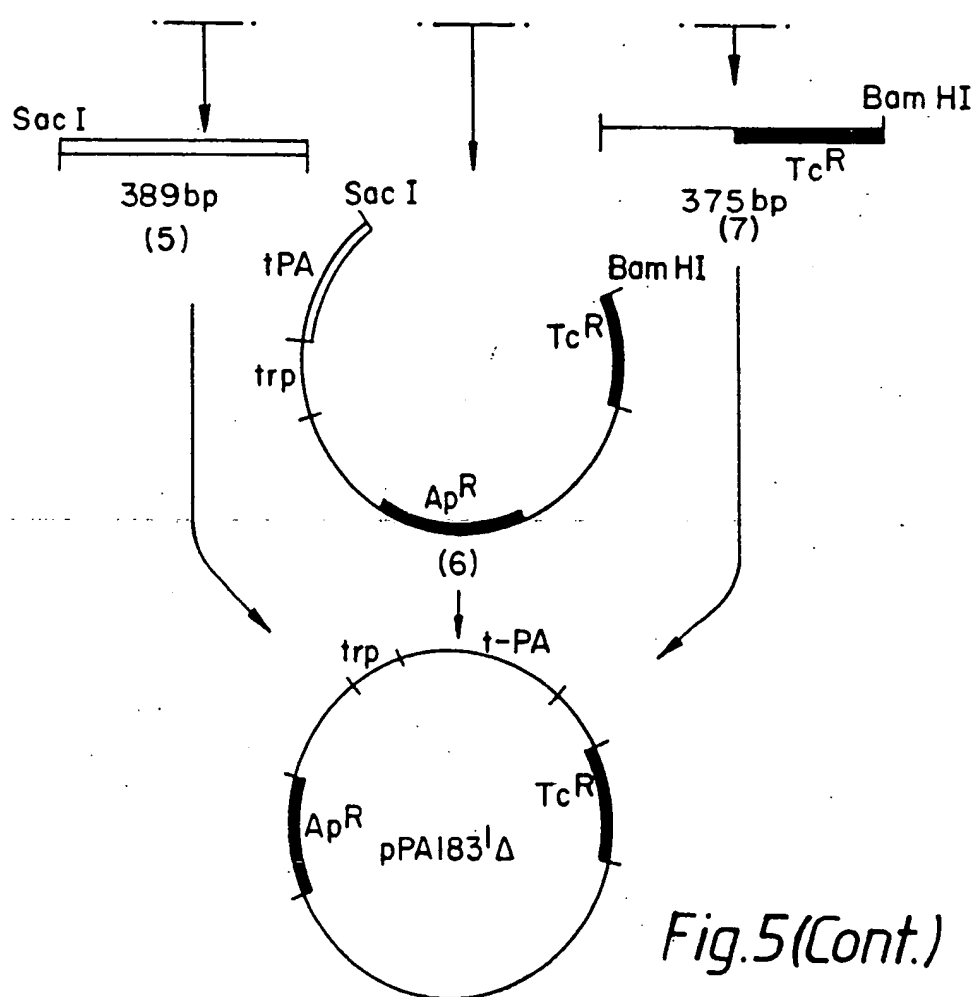


Fig.5(Cont.)

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Fig.6.

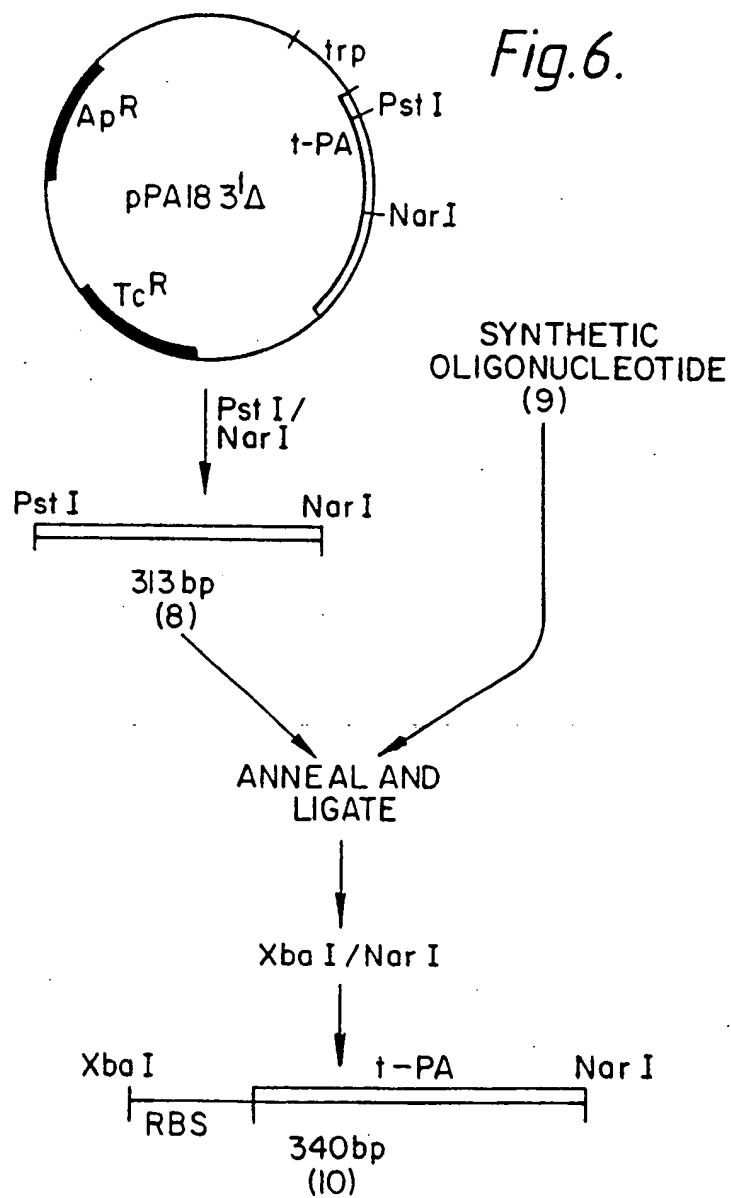
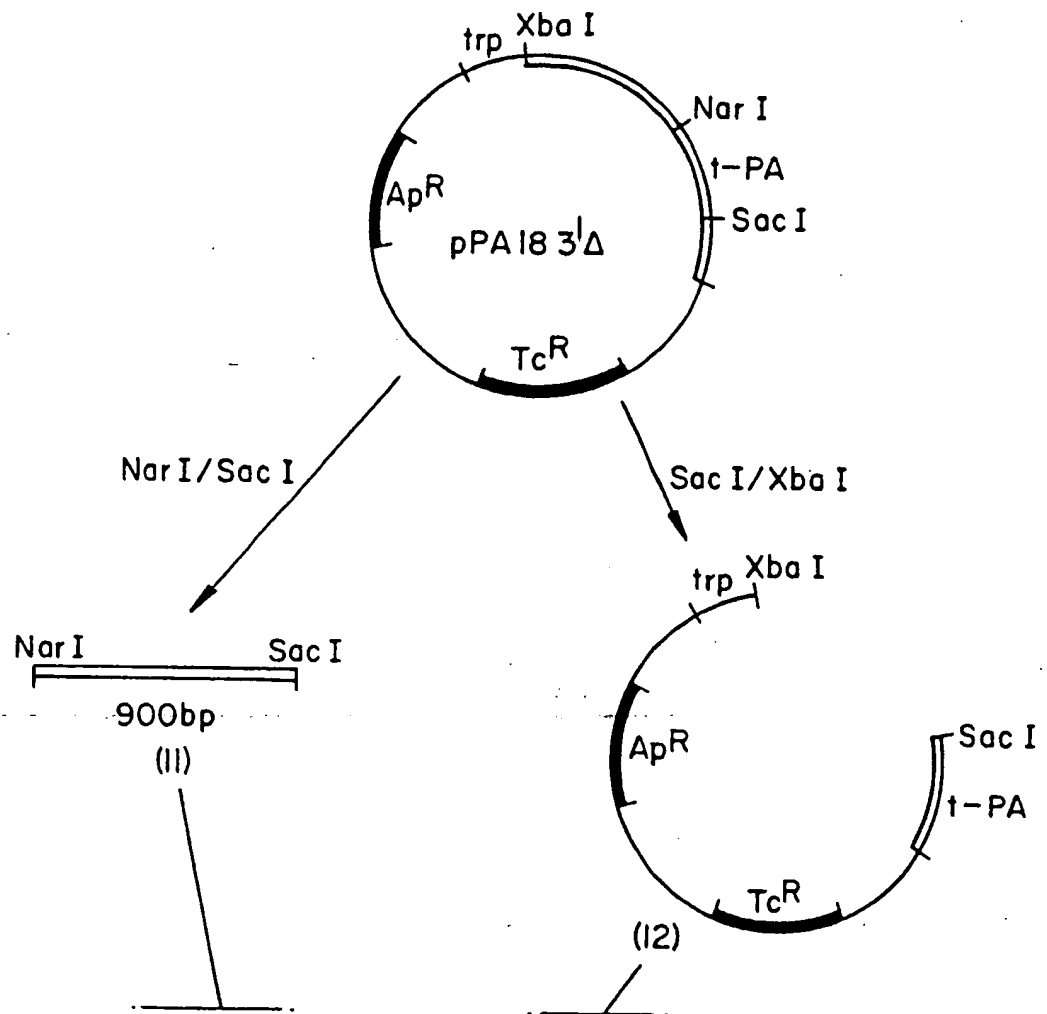
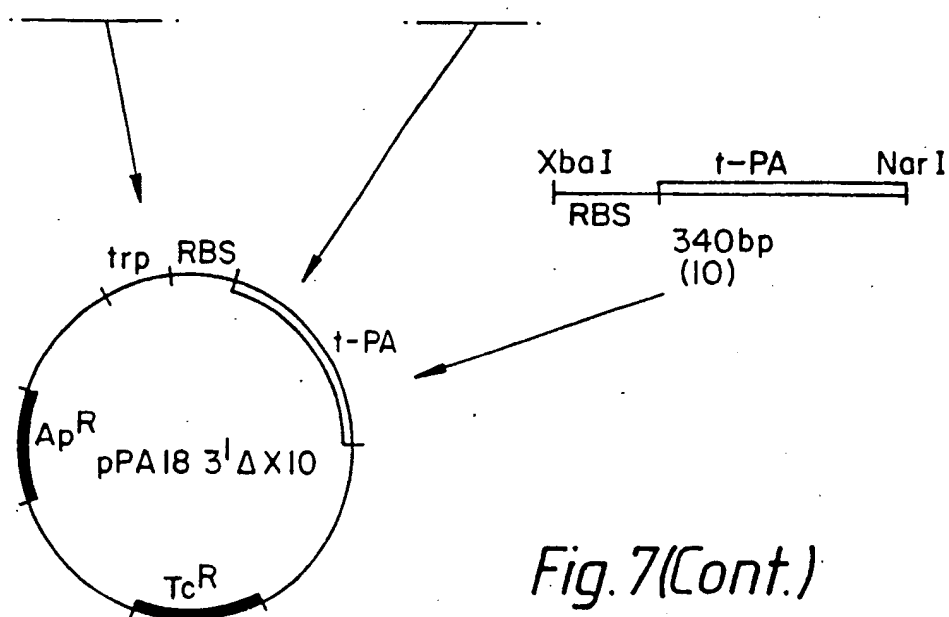


Fig. 7.



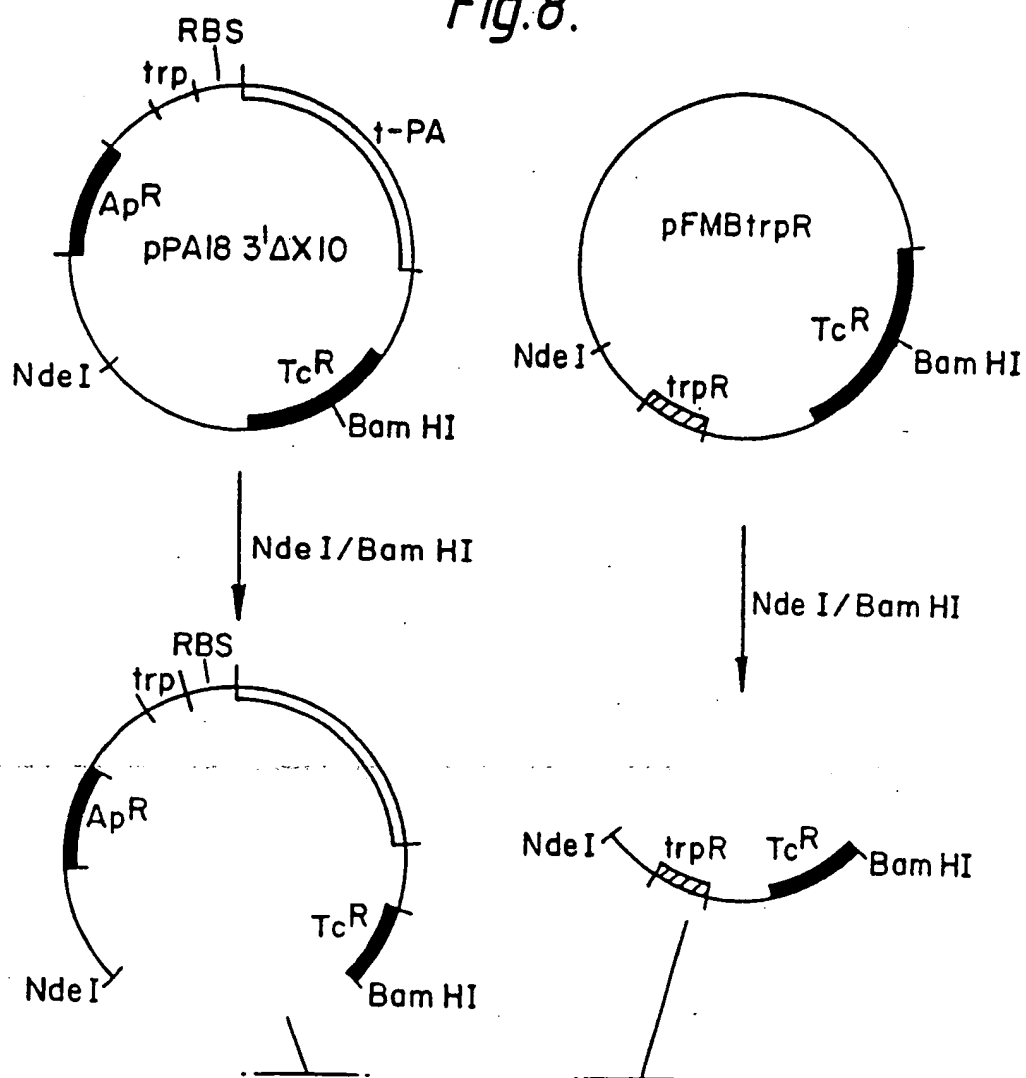
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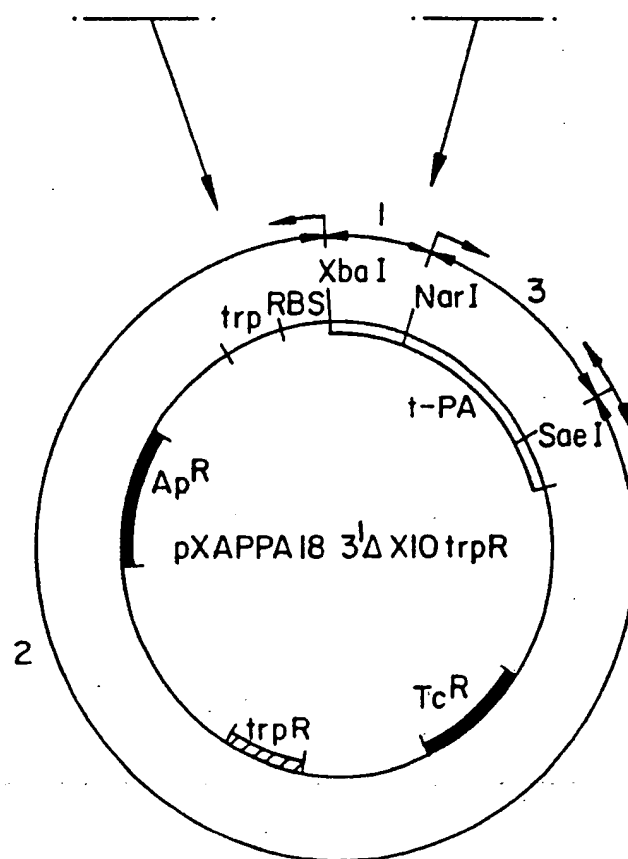


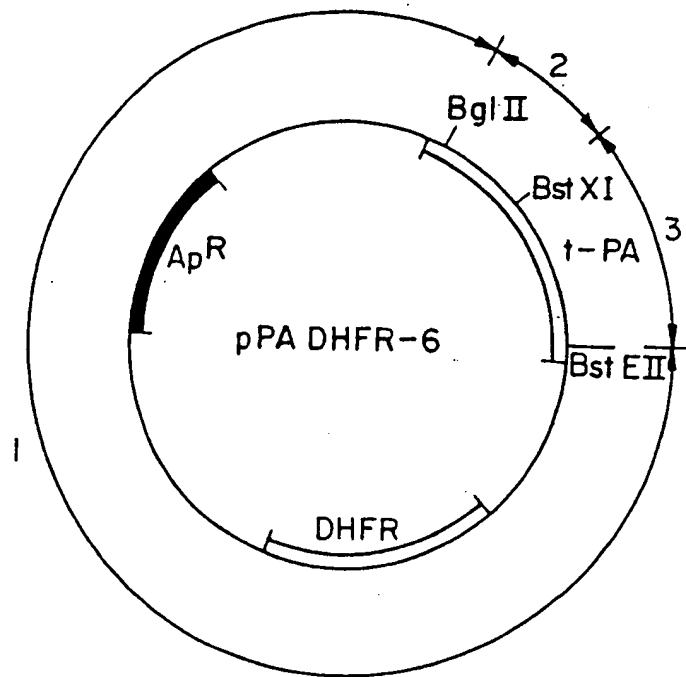
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Fig. 8.

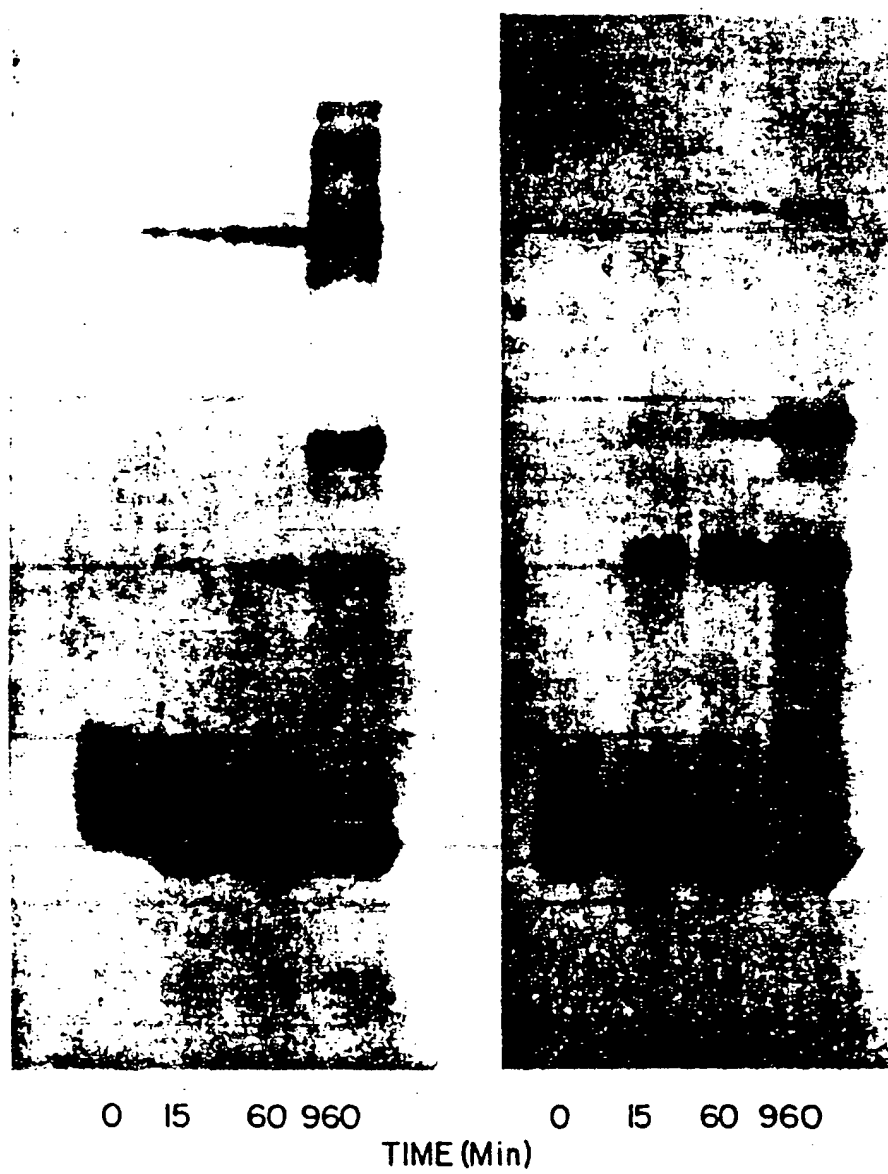


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*Fig.8(Cont.)*

*Fig. 9.*

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*Fig.10.*

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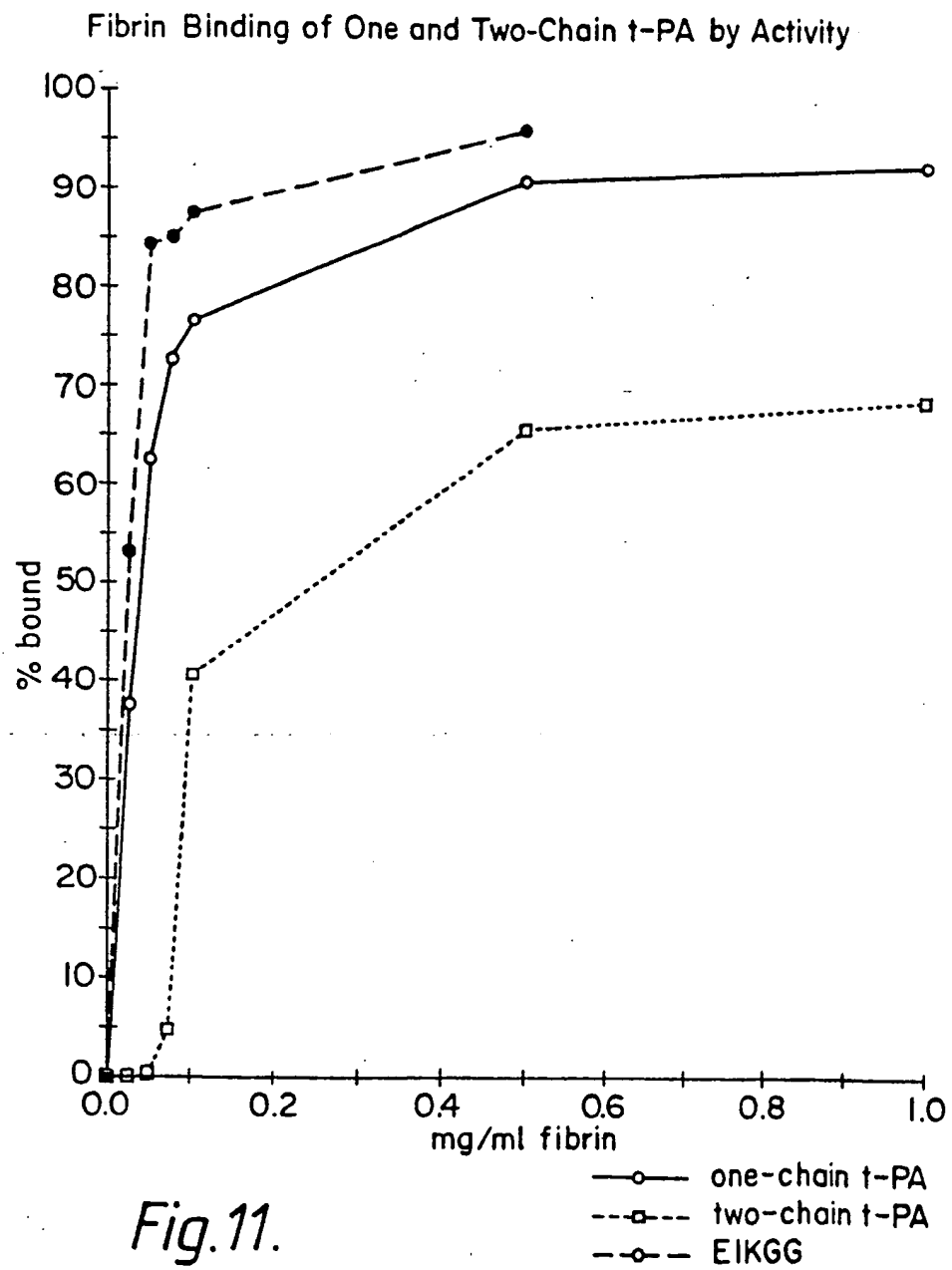
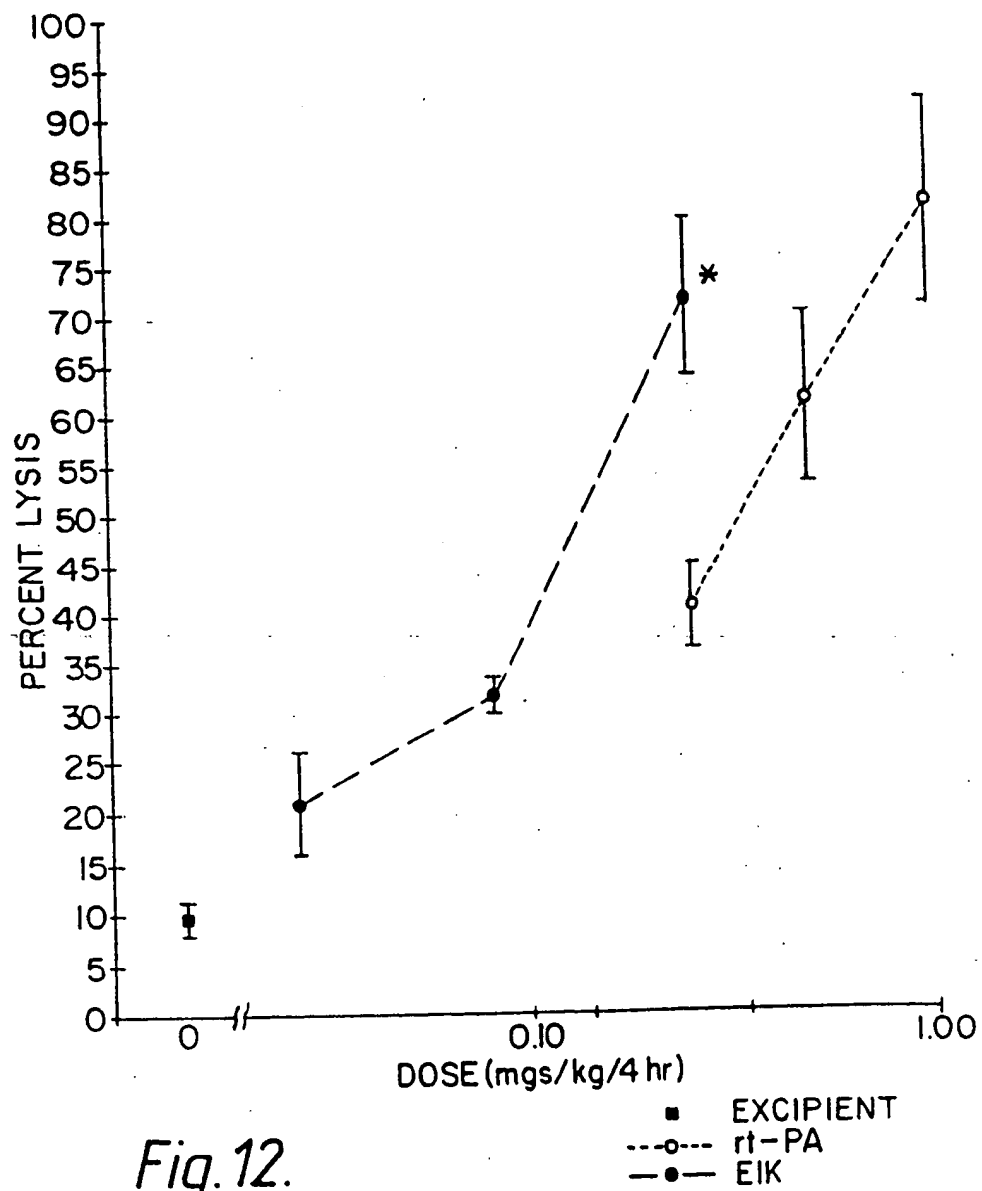


Fig.11.

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## EIK / TPA: IN VIVO CLOT LYSIS



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**EUROPEAN PATENT APPLICATION**

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71 Applicant: **GENENTECH, INC.**  
460 Point San Bruno Boulevard  
South San Francisco California 94080(US)

72 Inventor: **Heyneker, Herbert Louis**  
501 Roe Hampton Road  
Hillsborough California 94010(US)

72 Inventor: **Vehar, Gordon Allen**  
14 Maple Way  
San Carlos California 94070(US)

74 Representative: **Armitage, Ian Michael et al,**  
**MEWBURN ELLIS & CO. 2/3 Cursitor Street**  
London EC4A 1BQ(GB)

54 Human tissue plasminogen activator mutants, methods and intermediates therefor, and compositions using such mutants.

57 Biologically active mutant tissue plasminogen activators are disclosed wherein site directed mutagenesis, for example, of a two-chain activation site, especially in the region of residues 270-279, renders said mutants resistant to conversion to the two-chain form.

Particularly preferred mutations are substitution of arginine at residue 275 and/or isoleucine at residue 276.



European Patent  
Office

# EUROPEAN SEARCH REPORT

0199574

Application number

EP 86 30 2980

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D, Y	EP-A-0 093 619 (GENENTECH INC.)  * Page 9, lines 19-33; page 10, lines 19-29; page 51, lines 16-24 *	1-5, 8, 9, 12-15	C 12 N 15/00 C 12 N 9/64 A 61 K 37/54 C 12 N 1/20 C 12 N 5/00
Y	--- NATURE, vol. 301, no. 5897, January 1983, pages 214-221, Macmillan Journals Ltd, London, GB; D. PENNICA et al.: "Cloning and expression of human tissue-type plasminogen activator cDNA in E.coli" * Page 220, conclusion *	1-5, 8, 9, 12-15	
A	--- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 80, no. 2, January 1983, pages 349-352, Washington, US; T. EDLUND et al.: "Isolation of cDNA sequences coding for a part of human tissue plasminogen activator" * Pages 351, 352, discussion *	1	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)  C 12 N
P, X	--- WO-A-8 601 538 (BIOGEN N.V.) * Abstract; page 4, line 25 - page 5, line 9; claim 1 *	1, 7, 12	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-04-1987	Examiner YEATS S.M.
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